BACTERIAL AND MYCOTIC INFECTIONS OF MAN

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BACTERIAL AND MYCOTIC INFECTIONS OF MAN

Edited by

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Fourth Edition



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Preface to the Fourth Edition

The extensive revisions made in this the fourth edition of *Bacterial and Mycotic Infections of Man* go far beyond bringing up to date the factual contents of earlier editions While the general structure and the purpose of the book remain unchanged much new material has been added to strengthen the theoretical basis of medical microbiology and to enlarge the understanding of its application to disease processes

The first chapters deal with the historical evolutionary and ecologic aspects of microbiologic sciences They are followed by a study in some depth of modern microbial morphology genetics and metabolism then of the constituents and the properties of microorganisms more directly relevant to their pathogenicity

The second part of the book deals with host response to infection Host resistance mechanisms are first considered with regard to the body as a whole then with special emphasis on phagocytic cells and humoral agencies Hypersensitivity phenomena and physiologic and pathologic responses to microbial invasion complete the discussion of the general disorders encountered in infectious processes

Chapters 14 through 37 deal with specific groups of microorganisms Each of these chapters has been rewritten and brought up to date One new chapter has been added to cover atypical pneumonia caused by Mycoplasma an entity which had not been defined at the time the previous edition was published

The book ends with three new chapters devoted to the general and practical problems of epidemiology prevention and therapy

In the previous edition an effort had been made to restrict the number of references in order to save space The experience of those who have used the book makes it clear that this was false economy therefore we have greatly enlarged the list of pertinent references at the end of each chapter

The first three editions of *Bacterial and Mycotic Infections of Man* had been published under the sponsorship of the National Foundation While the present fourth edition is being published independently of it we wish to thank the Foundation for its early encouragement to prepare a book focused on the needs of the student the physician and the investigator interested in the problems of microbial diseases

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Preface to the First Edition

This volume was designed to convey to the medical student—and we hope also to the practitioner of medicine—some knowledge of the bacteria actinomycetes and molds pathogenic for man as well as of the phenomena which characterize the infectious process Infections caused by viruses and rickettsiae are treated in a companion volume edited by Dr T M Rivers

Medical microbiology is the study of host parasite relationships and not that of microorganisms alone considered as independent living agents It is concerned with those aspects of the structure and the properties of parasites which play a part in their pathogenic behavior and with the multiple manifestations of the response of the invaded host to their constituents and products The general chapters of this treatise are therefore devoted to the facts and the problems concerning parasite and host which have a bearing—often immediate but at times only potential and remote—on infectious disease

A few words may be necessary to justify the order in which the different pathogenic agents are described in subsequent chapters This order was adopted to illustrate by the extensive treatment of a few selected examples the multiple facets of the problem of infection Thus the diphtheria bacillus is discussed first to introduce the concept of toxemia and of antitoxic immunity As a counterpart pneumococcus infections are then selected to emphasize the problems of antibacterial immunity Streptococci on the other hand lend themselves to the demonstration that a given microbial agent can exhibit multiple pathogenic potentialities and that tissues can respond in many different ways to its presence Tuberculosis illustrates particularly well the acute (exudative) and chronic (proliferative) pathologic processes accompanying infection and the altered reactivity of the body (allergy) which results from previous exposure to the bacillus All these aspects of the infectious process appear in more or less modified form in the other microbial diseases and give to each of them its peculiar character

This treatise is the result of the co operative effort of many experts and naturally reflects their individual outlooks I wish to thank them all in particular for their willingness to aim at some measure of uniformity in our common undertaking The National Foundation for Infantile Paralysis has given generous financial support to the preparation of the book and shares with us the hope that it may contribute something to the understanding of the general problems of infection

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Contents

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1

The Evolution of Medical Microbiology

THE DISCOVERY OF MICROBIAL LIFE

Bacteriology is one of the very few biologic sciences *if not the only one which can* trace its origin to a single person one well documented observation and an instrument still *in existence On September 17 1683* Antonj van Leeuwenhoek (1632 1723) sent from Delft (Holland) where he lived to the Royal Society in London of which he was a correspondent the description of a new kind of animalcule smaller than any thing yet known he reported similar observations in another letter dated September 1692 Both letters were illustrated with drawings which leave no doubt that Leeuwenhoek had really seen bacteria in fact he clearly differentiated the main morphologic types which are still recognized today and he gave an excellent account of bacterial motility

Contrary to what is often stated Leeuwenhoek did not invent the *microscope Even* though the compound microscope was al*ready known in his time he never owned one* and made all his observations with a multiplicity of single lens microscopes of his own design and making He was an expert lens grinder and made numerous small lenses each mounted in a different way to suit particular purposes His simple instruments could give magnifications ranging from 40 to 275 diameters and he probably used some kind of darkfield illumination However these statements are conjectural because he was very secretive about his methods of observation he reported his findings in great details but *never describ d his technics* Fortunately he willed some of his instruments to the Royal Society which has preserved them ever since (Dobell 1932)

Another unique feature of the history of bacteriology is that it gives an unequivocal answer to a question much debated by his*torians of science The question is* whether science originates from sheer curiosity and *the desire to know more about the world* or from man s urge to achieve practical ends and *master his environment* The answer to this question is very clear in the case of Leeuwenhoek He earned his living as a draper and as an employee of the city of Delft Lens grinding and the exploration of the microscopic world were activities from *which he could not* possibly derive any material reward beyond the satisfaction of curiosity He worked alone had no sponsor and had no practical application in mind when he chanced to discover bacteria in an infusion of pepper and in material scraped from his teeth Entertainingly enough it was only a very few years ago—i e almost 300 years after his initial observations—that the presence of bacteria on teeth was first shown to be of practical importance as a cause of dental caries

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FIG 1 Antonj van Leeuwenhoek (1632 1723)

the size and the classification of bacteria as well as of their distribution and abundance in nature The development reached by such a purely descriptive kind of knowledge is illustrated for example in the textbook and the Atlas published in 1838 by Christian Ehrenberg (1795 1876) and in the short essay *Uber die Bacterien die Kleinsten Lebenden Wesen* published in 1872 by Ferdinand Cohn (1828 98) The more speculative aspects of bacteriologic science during the 18th and the 19th centuries revolved chiefly around the doctrines of heterogenesis and spontaneous generation

THE BIRTH OF EXPERIMENTAL MICROBIOLOGY

Throughout ancient civilizations and until late in the Renaissance it was taken for granted that plants and animals could be generated *de novo* under certain circumstances Although learned men eventually ceased to believe in the spontaneous generation of maggots and mice it long remained the general opinion that microbial life came into being through the spontaneous organization of organic substances in putrefying and fermenting materials Experimentation on this problem began in earnest around 1750

Some experimenters like the Irish priest John T Needham (1713 1781) claimed that they could bring about at will the creation of microscopic organisms in infusions previously sterilized by heat In contrast another priest Lazaro Spallanzani (1729 1799) maintained that living things however small never could be generated spontaneously from dead matter His contributions deserve special emphasis because they constitute some of the most important landmarks of experimental microbiology

Spallanzani recognized bacteria and grew them in sterilized media he observed that microbial life could occur in the absence of air and he discovered germs now called endospores of a greater resistance to heat than were the forms to which they gave rise Because of his rigid adherence to the experimental conditions required for testing the possibility of spontaneous generation he unfortunately did not inoculate his media on purpose and consequently failed to realize the general significance and technical possibilities of his work Otherwise he might well have become the founder of experimental microbiology

We cannot review here all the reasons and facts or rather pseudofacts which kept the doctrine of spontaneous generation alive until the second half of the 19th century nor can we mention the excellent experimental studies relevant to it which were carried out all over Europe during this period (For a discussion of these topics see Bulloch 1938 and Dubos 1950) However it may be said that the attitude of reasonable unprejudiced scientists on the subject was still uncertain in 1850 and that opinion was then evenly divided between the protagonists and the opponents of spontaneous generation Shortly after that time Louis Pasteur (1822 1895) entered the arena and his work convinced the scientific world that bacteria and molds never generate spontaneously With extraordinary thoroughness and elegance Pasteur demonstrated that no growth of any sort ever develops in sterilized media when all precautions are taken to keep out the microorganisms present in the surrounding air or on nearby objects But he showed also that the precautions to be taken were most exacting and that earlier investigators who had thought that they had observed spontaneous generation had not

been aware of the difficulties involved Among the many types of experiments that Pasteur designed to rule out spontaneous generation one is worth some emphasis because of its very simplicity and decisiveness and also because it finally settled the issue

A fermentable fluid was put into a flask the long neck of which was then heated and drawn into the form of an S tube (hence the name swan neck flask) When the liquid was boiled the vapor forced the air out through the orifice of the neck As the fluid became cool again the air slowly returned to the flask but was washed in the moisture that condensed in the curves of the neck after heating was interrupted Under these conditions any dust or particle carried by the air was trapped in the neck and the fluid in the flask remained clear sterile However when the neck of the flask was broken and the unwashed air was thus allowed to come into contact with the fluid microscopic life immediately began to develop Experimental difficulties arose from the fact that certain species of bacteria produce heat resistant spores In some of the early experiments these spores persisted in the fluid of the swan neck flasks that was presumed to have been sterilized by heating when they germinated they gave rise to bacterial growth even though access to outside air had been prevented Eventually these difficulties were overcome and Pasteur was able to prepare his flasks in such a manner that the broth remained sterile in them all Some of these flasks prepared almost 100 years ago are still in existence the fluid is as limpid as the day it was sterilized

For years the studies on spontaneous generation were carried out in an atmosphere of intense excitement and of passionate controversy because it was erroneously thought that the problem involved religious issues—a view which Pasteur denied strenuously In a famous lecture that he delivered at the Sorbonne in 1864 he expressed with eloquence his absolute conviction that the failure of bacteria to generate spontaneously was a purely scientific problem Presenting to his audience the swan neck flasks in which heated infusions had remained sterile in contact with natural air he exclaimed triumphantly

> I have taken my drop of water from the immensity of creation and I have taken it full of the elements appropriate to the development of microscopic organisms And I wait I watch I question it'—begging it to recommence for me the beautiful spectacle of the first creation But it is dumb dumb since these experiments were begun several years ago it is dumb because I have kept it sheltered from the only thing man does not know how to produce from the germs which float in the air from Life for Life is a germ and a germ is Life Never will the doctrine of spontaneous generation recover from the mortal blow of this simple experiment'

It is necessary to acknowledge here that present-day discussions on the origin of life seem to reopen once more the possibility of spontaneous generation As is well known the discovery that many viruses can be readily crystallized has fostered the hope that living processes can soon be identified with well defined chemical structures and processes originating from inanimate matter Many efforts are being made to imagine types of chemical reactions that would be self reproducing and thus exhibit one of the most distinctive properties of life Only time will tell to what extent today s discussions on the genesis of self-duplicating molecules of nucleic acids and of proteins really bear on the origin of life—a problem which demands philosophic definition at least as much as scientific description What is certain in any case is that modern scientific developments do not conflict with the view that all the microorganisms with which we have experience today—including the viruses—originate from structures fundamentally similar to themselves In fact it can be said that no constructive thinking on the origin of life was possible until the ancient doctrine of spontaneous generation had been discarded

One of the important by products of the studies on spontaneous generation beginning with Spallanzani s work was to provide the technical basis for experimental microbiology Exacting procedures had to be devised to destroy the microbes present in the systems under study and also to prevent the introduction of other microbes from the outside Sterilization and aseptic manipulation thus became the essential basis of microbiologic technics The necessity to grow



FIG 2 Louis Pasteur (1822 1895)

microbes in pure culture demanded investigation of their nutritional and physiologic requirements thus began theoretical microbiology which has now opened new vistas for general biology Incidental to the controversy on spontaneous generation there were discovered many important facts concerning the distribution of microbes in nature and in the various parts of the body knowledge of the natural history of microbial life made it possible to develop the technologic and medical aspects of microbiology It can be said truly that the controversies on spontaneous generation created the environment in which microbiology became aware of its problems and developed its methodology

The present essay is concerned not with the general history of microbiologic sciences but only with their medical aspects Nevertheless it seems to be important to emphasize at the outset that medical microbiology emerged in large part from studies apparently unrelated to problems of health and disease

THE GERM THEORY

The evolution of experimental microbiology has been influenced profoundly by the fact that its founder Louis Pasteur received his advanced training not in biology but in theoretical physics and chemistry (Vallery Radot 1911 Dubos 1950) In fact Pasteur s first great achievements dealt with the relation between the morphology of crystals and their ability to rotate the plane of polarized light One of the unexpected consequences of his chemical work was the discovery in 1848 that a certain mold could utilize the dextrorotatory form of tartaric acid but not its levorotatory isomer This apparently small fact determined once and for all Pasteur s subsequent scientific life by convincing him that microbes can bring about profound transformations of organic matter and that they are extraordinarily selective and specific in their activities He soon applied this general concept to the microbial transformation of sugar into lactic acid alcohol or other organic derivatives and of alcohol into acetic acid a few years later he observed that sugar was converted into butyric acid in the absence of air and thereby he discovered anaerobic life He demonstrated that each of the chemical transformations he had studied was carried out by one particular kind of microbe having very specific nutritional and environmental requirements It was in the course of these studies that he was led to rule out the accidental introduction and the *de novo* production of microbial life in fermenting fluids and thus to disprove the theory of spontaneous generation

The chemical approach to the study of microbial life immediately led Pasteur to apply his newly acquired knowledge to various fermentation industries in particular to the production of vinegar wine and beer One of the by products of his technologic activities was the technic of controlled heating for the preservation of beverages foodstuffs and other organic materials which are perishable because they are attacked readily by microbial life This technic now called

pasteurization in his honor proved of course to have large medical applications but the broader and more important relevance of Pasteur s work to medicine came from the fact that he perceived intuitively from the very beginning that the germ theory would be applicable to problems of disease In fact he expressed this conviction in his very first paper on fermentation namely the *Memoire sur la fermentation appelee lactique* which he published in 1857 when he was 35 years old (Pasteur 1857) This paper can properly be regarded as the manifesto of experimental microbiology therefore it seems justifiable to present here an outline of its contents

The 1857 *Memoire* was an attempt to demonstrate that the so-called lactic acid ferment is not just an amorphous chemical substance as was then believed but is constituted instead of organized living microscopic bodies which all resemble one another Furthermore Pasteur emphasized that the degree of acidity or alkalinity of the fermenting solutions has very profound effects on the activity of the various kinds of ferments Thus yeast produces alcohol most rapidly in an acid solution whereas the lactic acid ferment is most active at neutrality He even recognized—for the first time—the activity of certain antiseptics Onion juice he found inhibited the action of yeast but not of the lactic acid ferment

The association of a specific microbe with each fermentation the disproportion between the weight of microbe produced and the weight of matter transformed the competition between two organisms simultaneously invading the same medium resulting in the dominance of the one better adapted to the cultural conditions—all these ideas which were to grow into a large body of science and technology are forcefully set forth in the preliminary 1857 paper For this reason the *Memoire* can truly be regarded as one of the most important landmarks in the history of biochemical and biologic sciences Its fundamental spirit can be summarized in Pasteur s words

> The purity of a ferment its homogeneity its free unrestrained development from foodstuffs well adapted to its individual nature these are some of the conditions which are essential for good fermentation



Fig 3 Robert Koch (1843 1910)

With extraordinary foresight Pasteur suggested that the same principles might explain the genesis of many forms of disease and that particular kinds of microbes would be found to be responsible for particular kinds of maladies As he stated elsewhere in prophetic words

> There are similarities between the diseases of animals or man and the diseases of beer and wine If fermentations were diseases one could speak of epidemics of fermentation

THE DISTANT ORIGINS AND THE SCIENTIFIC BEGINNINGS OF MEDICAL MICROBIOLOGY

It is customary to state that medical microbiology began in 1876 when Robert Koch (1843 1910) and Pasteur demonstrated almost simultaneously and unknown to each other that the disease anthrax can be pro

duced experimentally by injecting into animals small amounts of culture of a particular microbial species (*Bacillus anthracis*) Before the significance of this laboratory achievement can be discussed adequately it is essential to emphasize that the *concept* of infection was not new in 1876 It had evolved progressively over many centuries through large numbers of careful observations shrewd hunches and bold experiments Only a few of the very many steps in this long historical development can be mentioned here

It has long been recognized of course that certain diseases are catching and that some confer protection against a second attack but although the idea of contagion passing from one individual to another was evident in diseases such as plague and syphilis it was missed completely in many other conditions Girolamo Fracastoro (1478 1553) was probably the first to state explicitly in his book *De Contagione* (1546) that infection itself is composed of minute and insensible particles and proceeds from them He wondered whether all contagion might not be a putrefaction and recognized that the infection is the same for him who has received and who has given the infection

Of course much caution is required in interpreting the early speculative writings on infectious disease because the words used by ancient authors can but rarely be applied in their modern meaning However a few concepts emerge clearly from the past Thus A Kircher (1602 80) probably the first to make direct microscopic studies of diseased tissue examined putrefying materials and even blood from plague patients On the basis of his observations he postulated that animated corpuscles scatter the seeds of contagion and that it is difficult to wash them away Therefore he recommended burning household goods and clothing suspected of carrying contagion The specificity of contagious diseases was expressed forcefully by Thomas Fuller (1654 1734) who said that one type of disease could not change into another any more than a Hen can breed a Duck he emphasized further that consequently one Sort cannot be a preservative against any other Sort

One of the outstanding experiments of the premicrobial era was carried out by John Hunter (1728 93) who inoculated himself with syphilis using material from a case which he assumed erroneously to be simple gonorrhea In addition to the tragic consequences for his own life Hunter s experiment was responsible for establishing the belief which persisted for many years that gonorrhea and syphilis were the same disease Of great importance also were the writings by Charles White (1728 1813) in 1773 Oliver Wendell Holmes (1809 94) in 1843 and I P Semmelweis (1818 65) in 1846, concerning the role of cleanliness and other practical sanitary methods in the prevention of puerperal sepsis and of blood poisoning from putrid wounds

Agostino Bassi (1773 1856) demonstrated in 1836 that a fungus was the primary cause of a silkworm disease he formulated the germ theory of disease in very explicit terms In 1839 Johann Schonlein showed that the fungus now known as *Achorion schonleinii* is always present in favus and in 1844 *Trichophyton tonsurans* was established as the cause of *herpes tonsurans* The parasitic role of fungi gained wide acceptance through the publication in 1853 of Robin s *Histoire Naturelle des Vegetaux Parasites*

In 1840 the pathologist Jakob Henle (1809 85) drew up a statement of the conditions which would be required to provide proof of a causal relationship between microbes and the diseases of animals and man These conditions are now known as Koch postulates although they do not appear in concise form in the writings of Robert Koch who had been Henle s student

The first convincing experimental observations on a bacterial cause of disease was made by C J Davaine (1812 82) who detected as early as 1850 minute rods which he called infusoria in the blood of sheep dying of anthrax Stimulated by Pasteur Davaine returned to this discovery and described his results in 1864 He transferred the disease to healthy animals by infecting them with blood containing the characteristic rods infection occurred even when the blood was diluted a million times In 1865 J A Villemin (1827 92) showed that tuberculosis also could be transmitted at will by injecting tuberculous matter from tuberculous human beings to animals and from

one tuberculous animal to another From 1865 to 1869 Pasteur turned his attention to pebrine a disease of silkworms and demonstrated its parasitic nature moreover he developed practical control technics based on the detection of the causative protozoon in the tissues of infected worms These technics were so effective that they saved the European silkworm industry which had been threatened with destruction by the protozoon

The most spectacular contribution to the germ theory of disease before 1876 came from the English surgeon Joseph Lister (1827 1912) (Godlee 1918) Lister was familiar with Pasteur s publications on the ubiquitous presence of microbes in the air and on their chemical activities and he postulated that this knowledge might help to explain many of the accidents then so commonly encountered in surgical practice He surmised that the microbes surrounding the patient at the time of operation could fall on open wounds and thus be responsible for putrefaction and sepsis Acting on this theory he made it a practice to spray solutions of phenol around his patients during surgery and thus greatly decreased the rate of fatal infections Lister s achievement opened the way for modern surgery and constitutes unquestionably the first conscious application of the germ theory of disease to an important problem of human medicine

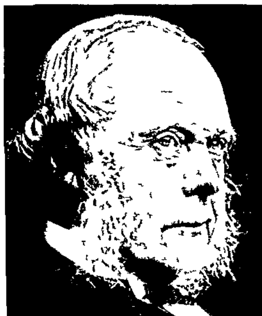


Fig 4 Baron Joseph Lister (1827 1912) (Godlee Lord Lister London Macmillan)

Thus it is certain that infection and microbial etiology were not new concepts when Koch and Pasteur first published their studies on anthrax However these concepts were then ignored or even scornfully rejected by most medical scientists and practicing physicians Even Lister s achievement great though it was did not impress the medical world physicians failed to recognize its relevance to other disease conditions Surprising as it may seem to us the idea that microscopic organisms could cause damage to the body seemed then to be incongruous and contrary to common sense Furthermore the skeptics pointed to the fact that many kinds of microbes are always present in large numbers in the body of healthy men and animals and therefore could not be of great significance in the causation of disease

The great merit of Koch s and Pasteur s experimental studies on anthrax was to prove irrefutably that a certain kind of disease always occurred when a few microbes of the proper kind were introduced into the body and only then Causal relationship was proved by demonstrating that anthrax bacilli which had been made to multiply outside the body for large numbers of generations—in serum urine or broth—retained nevertheless the ability to cause in animals the particular kind of disease with which they had first been found to be associated The validity of the evidence adduced by Koch and Pasteur rested entirely on the exacting nature of the technics they used for isolating the bacteria for cultivating them in pure cultures and for making sure of their identity

Robert Koch s greatness came precisely from the immense skill and ingenuity that he displayed in devising new bacteriologic technics and from the austere discipline with which he applied them to etiologic problems (Bochalli 1954) Suffice it to mention that the plating of cultures on agar surfaces the photomicrography of bacteria and the use of aniline dyes for demonstrating them microscopically were all methods developed in his laboratory

After he had isolated the anthrax bacillus in pure culture and demonstrated its infectivity Koch proceeded to study traumatic infectious diseases and developed methods for the separation of pure cultures from mixed infections (1878 1881) However his most spectacular achievements were the isolation and the cultivation of the tubercle bacillus in 1882 and of the cholera vibrio in 1883 The dramatic character of these diseases and their great prevalence during the 19th century made of Koch a world hero he was treated as a demigod when he traveled to Japan and a shrine was erected in his name

The decades that followed the birth of the germ theory saw the isolation and the characterization of most of the bacterial agents of disease Progress was very rapid because the experimental philosophy and methods developed by Pasteur and Koch were applicable to many types of infections with only minor changes of detail However more profound modifications became necessary when emphasis shifted from bacterial to rickettsial and viral infections Rickettsia and viruses being unable to grow in lifeless media their isolation and cultivation required the development of new methods based chiefly on the use of fertilized eggs and tissue cultures (see the companion volume of this series edited by Tamm and Horsfall)

The era opened by Pasteur Lister and Koch has been properly called the Golden Era of medical microbiology and it probably represents the greatest single contribution so far made to the theory and the practice of medicine There is no doubt that the stimulus to this phenomenal advance was the establishment of the bacterial cause of disease which provided a scientific basis for etiologic diagnosis specific therapy and preventive medicine The profound reformation of medical thought brought about by bacteriologic knowledge was achieved against violent opposition but experimental medicine emerged from the bitter struggle much strengthened and enlarged Experimental microbiology thus constituted more than a useful helping hand to medicine it was the guiding finger as well It wrought such changes in human health that its contributions to human welfare are at least equal to those of any other science Diseases such as typhoid fever diphtheria tuberculosis and pneumonia have been reduced from scourges with a high mortality to manageable conditions surgery was lifted out of the despondency of laudable pus into the safety of asepsis The great epidemics of the past remain a threat only where the required precautions are neglected

THE STRUCTURE AND THE PROPERTIES OF MICROORGANISMS

One of the first tasks faced by medical microbiologists was to devise technics for the cultivation the differentiation and the identification of the agents of disease Much of the microbiologic literature published around the turn of the century deals with the development of selective culture media the definition of physiologic activities (especially the ability to attack various sugars alcohols and amino acids) the manner of growth in broth on agar in gelatin aerobically or anaerobically etc From the scientific point of view this literature is rather dull yet it provided the kind of information on which much of microbiologic taxonomy and etiologic diagnosis still depends today Fortunately a broader kind of knowledge continued to develop along with this prosaic though essential type of information Progressively the study of microbes as living entities moved into the main channels of biologic thought

To the biologist of the 19th century bacteria had first appeared to be so primitive as to be at the very threshold of life Speaking of what he considered the smallest and at the same time the simplest and lowest of all living forms Ferdinand Cohn (1828 98) asserted in 1872 They form the boundary line of life beyond them life does not exist so far at least as our microscopic expedients reach and these are not small The minute dimensions of bacteria were considered by many to be incompatible with any significant morphologic differentiation it encouraged the chemist to treat the bacterial cell as a simple colloidal system and as a bag of enzymes This assumed primitiveness of structure appeared to be confirmed in biochemical terms when Serge Winogradsky (1856 1953) announced in 1887 that cer

tain microorganisms—those of the auto trophic group—could synthesize their proto plasm from mineral salts and carbon dioxide utilizing for the reduction of the latter the energy released by the oxidation of inorganic substances—ammonia nitrites sulfur hy drogen etc Could not this most primitive biochemical expression of life the production of organic matter from inorganic substances be considered as the beginning of *life* on earth?

Cytologic studies were quick to dispel any illusion as to the structural simplicity of bac teria Differential staining reactions studies of sporulation motility osmotic behavior etc revealed the existence in bacteria of various kinds of plasma membranes cell walls capsules intracellular bodies organs of locomotion etc which give to each bac terial type a characteristic complexity Fur thermore the biochemist soon came to rec ognize in microbial life the same type of chemical reactions biocatalysts metabolic channels and products which occur in the highest organisms Even the autotrophic bac teria those primitive beings capable of synthesizing life from the atmosphere and the rock are now known to operate through the same elaborate mechanisms found in the most evolved metabolic types

Many books published during the past 100 years on the structure and the properties of microorganisms make it easy to follow the development of knowledge in this field How ever the most striking impression gained from such a survey is not so much the in crease in amount of knowledge as the quali tative change in attitude The change was compelled by the progressive discovery that microbial life and its role in health and dis ease cannot be understood without consider ation of all aspects of biology—the most de tailed in terms of chemical or genetic analysis as well as the most complex in terms of cel lular and ecologic integration Chapters 3 and 5 of the present volume illustrate the recent growth of knowledge concerning the metabolic and structural complexities of mi croorganisms Chapters 2 and 38 reveal that microorganisms display the widest pos sible range of interrelationships with other living things from destructive parasitism to complete symbiosis—and that these inter relationships have a Darwinian evolutionary basis The knowledge concerning bacterio phage will serve as one example among many others that could have been selected to illustrate that points of view concerning microscopic life continue to change even in our time

The discovery of bacteriophage lysis by d Herelle and Twort appeared at first sight to confront biologists with the most primitive manifestation of life indeed with a phe nomenon in which it seemed that biology and chemistry could no longer be differentiated For a whole generation bacteriophage was considered as a substance to be crystallized and defined by purely chemical methods and the lysis of bacteria that it causes was as sumed to be a straightforward enzymatic process However recent discoveries have shattered these illusions Electron microscopy shows that bacteriophages are viruses with highly complex organizations made up of several morphologic structures each with a different chemical composition and a differ ent function bacterial lysis is the outcome of a complex infectious process during which the viruses multiply at the expense of meta bolic processes in the bacterial cells which they parasitize Bacteriophages can enter into various types of associations with the proper kinds of bacteria destroying them in some cases but also capable of endowing them with new potentialities under other circum stances Finally bacteriophages can mutate and thus undergo hereditary changes akin to evolutionary adaptation Such complexity is of course incompatible with the view that bacteria and bacteriophages are at the threshold of life

Thus two independent trends can be rec ognized in the development of knowledge concerning microorganisms as living entities On the one hand microbial structure and metabolism are being defined in more and more precise chemical terms on the other hand evidence is accumulating that even the smallest microorganism is a highly complex structure and that its living processes involve adaptive responses to the total environment as well as integration with other living things Microbiology has found its way back into general biology Furthermore the extraor dinary plasticity of microorganisms the ra

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Analysis of the mechanisms through which the body develops resistance against infec tion has proceeded along two independent channels One group of studies was focused on the role played by various kinds of tissue cells in resistance to infection another group emphasized in contrast the protective activ ity of blood serum and other humors For many years a violent polemic raged between the protagonists of the cellular theory led by Elie Metchnikoff (1845 1916) and the pro tagonists of the humoral theory led by Emil Behring (1854 1917) and Richard Pfeiffer (1858 1945) Progressively however the truth emerged that cellular and humoral fac tors both play a role and indeed often act jointly The modern position in this regard is presented in Chapter 8 For the sake of convenience it will be helpful to consider separately the development of knowledge concerning the cellular and the humoral mechanisms of defense

Between 1870 and 1877 several patholo gists and microbiologists had reported that bacteria were often seen in the leukocytes of pus they concluded from such observations that these cells provide a suitable lodgement for microorganisms and help them to become distributed throughout the body Metchnikoff placed a radically different interpretation on these facts Observations in invertebrates and vertebrates convinced him that ameboid (mesodermal) cells commonly destroy the microbes that they ingest for this reason he called them phagocytes (devouring cells) and concluded that they protect the body against infection Indeed he came to regard phago cytosis—the scavenging activity of macro phages and microphages—as the most im portant mechanisms of defense (1883) This view was strengthened through the demon stration by Denys and Leclef in 1845 that immunization greatly enhances phagocytic activity

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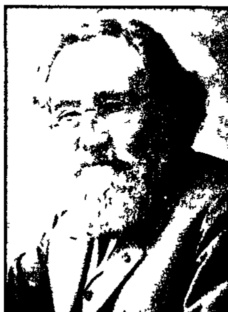


FIG 6 Elie Metchnikoff (1845 1916)

E Roux and A Yersin in 1887 88 and tetanus toxin by Knud Faber in 1890 The same year Behring produced diphtheria anti toxin and then tetanus antitoxin (in associa tion with S Kitasato) Further work by Behring and E Wernicke then by Roux and Martin soon made it possible to treat diph theria in man by antitoxin produced in horses The development by Paul Ehrlich (1854 1915) of methods for the standardiza tion of toxins and antitoxins (1896) pro vided a quantitative basis for therapy and for the analysis of antitoxic action During the late 1880 s and early 1890 s several other findings provided additional support for the humoral doctrine of immunity

It will be noted from the preceding para graph that the fundamental discoveries con cerning toxins and antitoxins were all made within a very few years This rapid accumu lation of new findings has remained to this day a characteristic of the science of humoral immunity—a consequence of the fact that it is much easier to experiment with serum and other body fluids than with living tissue cells A number of discoveries with their respec tive dates will be listed in the following para graph to illustrate further the rapidity with which the new science developed

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FIG 5 Oswald Theodore Avery (1877 1955)

pidity with which they respond to the environment either by hereditary genetic variations or by reversible phenotypic modifications makes them ideal objects for studying some of the problems which are common to all living things It is worth noting here for example that the lifelong dedication of Oswald Theodore Avery (1877 1955) to the study of pneumococci has paved the way for two large developments of modern biologic research namely the immunochemical analysis of cellular structure and the role of deoxyribonucleic acids in the transmission of hereditary characteristics Microbes truly appear as the most versatile tools of the general biologist

THE RESPONSE OF THE BODY TO INFECTION

From the most ancient times and even among extremely primitive people methods have been developed empirically to increase resistance to noxious influences and infectious agents In several tropical countries people expose themselves deliberately to various venoms in order to develop resistance to snake bites the word mithridization recalls that King Mithridates had made it a practice to ingest small amounts of several poisons in order to protect himself from poisoning by his enemies Ancient physicians of the Near East had learned that they could protect young women from the disfiguring effect of Aleppo boils by inoculating them on the thigh early in life with some of the infectious material Variolation the deliberate infection with smallpox under controlled favorable circumstances had been practiced widely in Eastern countries long before the technic was introduced into England in the 18th century The demonstration by E Jenner (1749 1823) in 1796 that resistance to smallpox could be induced by injecting into man material from naturally acquired cowpox was the substantiation of a popular belief The technic came to be called vaccination from vacca the cow and was immediately a triumphant success Wherever it was applied systematically it reduced smallpox from a universal and often fatal disease to a medical rarity

Thus immunization had long been practiced without rational basis but it entered the domain of scientific medicine through *Pasteur s studies on experimental infections* In 1879 Pasteur observed accidentally that chickens which had been inoculated with an old culture of chicken cholera and had failed to develop the disease proved to be resistant when reinfected with a young and highly virulent culture of the same microbe Intuitively he recognized the analogy of this protective effect with that induced by cowpox against smallpox and he referred to the newly discovered phenomenon as vaccination in honor of Jenner Pasteur established the generality of the doctrine of specific immunization by developing vaccines against anthrax in 1881 and against swine erysipelas in 1882 he demonstrated their effectiveness and practical usefulness in spectacular field experiments *However it was his work on* rabies immunization (1888) that gained him international fame Rabies immunization occupies a place of immense importance in the

history of medical microbiology because it convinced the medical and the lay public that infectious diseases could be controlled by specific vaccines

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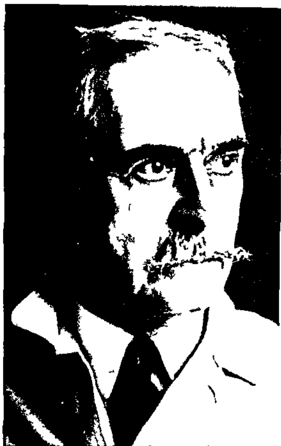


FIG 7 Karl Landsteiner (1868 1943)

1886 complement (alexin) by H Buchner in 1889 the lytic action of immune serum on cholera vibrios by Pfeiffer in 1894

Around the turn of the century an immense number of reports were published describing the acquisition of new activities in the serum of animals immunized with various antigenic materials Only a very few of the representative contributions which thus gave rise to the derivative science of serology can be listed here In 1889 Charrin and Roger noticed that the cells of *B pyocyaneus* growing in immune serum lost their motility and clumped in agglomerated masses Durham (1896) and M Gruber (1897) then showed that such clumping by immune serum occurred specifically for each kind of bacterium Specific agglutination as the phenomenon came to be called soon was applied for diagnostic purposes in particular by F Widal for typhoid In 1898 Jules Bordet (1870 1961) showed that injection of red blood cells into animals also elicited the production of specific aglutinin and this discovery provided Karl Landsteiner (1868 1943) with a technic for the classification of blood groups (1900)

In 1898 Bordet observed that red cells were lysed by antiserum when fresh guinea pig serum (complement) was added to the system This discovery was followed by the recognition of many other examples of immune lysins Bordet and Gengou then demonstrated in 1901 that complement was removed or fixed in the course of serologic reactions and they worked out the complement fixation test as the most delicate technic for the detection of antigen antibody reactions In 1906 Wassermann Neisser and Bruck applied this technic to the serodiagnosis of syphilis

In 1897 Kraus observed that addition of specific antiserum to a clear filtrate of cholera vibrio culture resulted in the rapid production of a fine precipitate which aggregated into flocculi and settled to the bottom of the tube Similar findings were made with filtrates of cultures of typhoid and plague bacilli and the reaction proved to be highly specific to the kind of bacterium The serum components responsible for the reactions were called precipitins Furthermore it was soon found that highly specific precipitins could be produced not only for bacterial filtrates but also for many other organic substances especially proteins and polysaccharides The precipitin reaction was applied by Nuttal to the study of phylogenic relationships between mammalian species (1902 04) it permitted the serologic identification of blood stains and of adulterants in food needless to say it provided also a valuable tool for the analysis of immune processes

The first evidence of allergic reactions came from the discovery of tuberculin by Robert Koch in 1890 Koch s claim that tuberculin was useful in the treatment of tuberculosis proved to be erroneous nevertheless the tuberculin reaction turned out to be of immense importance as a highly specific diagnostic test of tuberculous infection In addition it became the prototype of the delayed type of allergy In rapid steps the field of allergy broadened through the discovery of anaphylaxis (Portier and Richet 1902)

of the Arthus reaction (Arthus and Breton 1903) of serum disease (von Pirquet and Schick 1905) and of the Prausnitz Kustner reaction (1921)

For a while agglutinins precipitins lysins and allergins were regarded as different substances elicited by the antigenic stimulus As was remarked facetiously every time an immunologist discovered a new phenomenon he attributed it to a new phenominin In reality however it soon became apparent that all immunologic phenomena had a similar chemical basis and that their specificity is due to some change in the body cells or serum constituents which make them fit the molecular structure of the respective antigen Paul Ehrlich was the first to formulate a theory accounting for these facts From his student days he had been obsessed with the belief that the selective staining of tissues by various dyes was an expression of chemical affinity he carried this line of thought into his analysis of the specific interactions between antigens and antibodies and into his formulation of the side chain theory and finally as we shall see later into his search for chemotherapeutic agents

The side chain theory was a pictorial as well as picturesque expression of the lock and key relationship which governs the chemical affinity between antigen and antibody Bordet elaborated on this theory by pointing out that while the specific adsorption of antigen or antibody had a chemical basis the final effects of the reaction were produced by a nonspecific action of electrolytes and a shift of electrical charges In 1907 S Arrhenius and T Madsen postulated that the antigen antibody reaction behaved as a *reversible reaction analogous to that between weak acid and weak base* and they concluded that free antigen free antibody and antigen antibody complex could coexist in the same mixture

The precise chemical formulation of immunologic specificity emerged from the monumental studies of Karl Landsteiner (1868 1943) whose classic monograph *The Specificity of Serological Reactions* (1944) gave its modern philosophy to the field The quantitative analysis of serologic reactions began with Ehrlich s standardization of toxin and antitoxin (1896) it was extended through the finding by Dean and Webb (1926) that antigen and antibody react in terms of relative proportions more or less independent of absolute concentrations This quantitative approach reached its now classic form in the studies of Michael Heidelberger who showed that mass action formulae can be developed for many types of serologic reactions

The complex nature of complement and the mechanism of its action are other aspects of immunologic science which are still in the process of definition (see Chaps 8 and 9) Finally it should be mentioned that the participation of neurohumoral mechanisms in immune processes has been emphasized by a few Russian workers but constitutes a field which is almost completely neglected at the present time

Each of the immunologic phenomena which have been mentioned has been used in the study of the constituents and products of the microbial agents of disease the main results of such antigenic analysis are described in the chapters of this book devoted to the various microbial groups Except in a few cases the analysis of in vitro serologic reactions has failed to provide a complete understanding of the processes which increase the resistance of the body to infection *Antitoxic immunity and the enhancement of* phagocytosis of pneumococci by antibodies directed against their capsular polysaccharides constitute clear and well worked-out examples of humoral immunity In contrast understanding of the mechanisms through which cells destroy the microorganisms that they engulf has not grown much since Metchnikoff s time indeed there is still much *doubt that cellular immunity really exists* as an independent entity

THE BIRTH OF CHEMOTHERAPY

As in the case of immunization many empirical methods had been developed for disinfecting contaminated materials and even for treating some microbial diseases long before the advent of the germ theory Thus fire quick lime and various kinds of fumigations have long been used for the purification of air or objects assumed to be contaminated Ipecacuanha root (containing

emetine) was considered as a specific in the treatment of dysentery before its effectiveness in amebiasis had been proved and especially cinchona bark (containing quinine) has been the standard remedy for malaria (as well as for other fevers) since it was introduced from South America in the 17th century Between 1750 and 1752 John Pringle presented before the Royal Society of London several papers describing Experiments Upon Septic and Antiseptic Substances thus introducing the term *antiseptic* into the scientific literature However as already mentioned the first *conscious* application of antisepsis based on the germ theory of disease was the use of phenol sprays by Lister in his surgical practice

The modern chemical theory of disinfection and chemotherapy has its origin in the experimental studies of Paul Ehrlich between 1905 and 1915 His biochemical approach was an extension of his views on antigen antibody reaction and can be summarized by two of his most famous aphorisms

> Only such substances can be anchored at any particular part of the organism which fit into the molecule of the recipient combination as a piece of mosaic fits into a certain pattern
>
> Antitoxins and antibacterial substances are so to speak charmed bullets which strike only those objects for whose destruction they have been produced



Fig 8 Paul Ehrlich (1854 1915) (*Marquardt* Paul Ehrlich Deutsche Verlags Anstalt)

Ehrlich was obsessed with the belief that there must exist synthetic chemicals which like dyes and antibodies have a selective affinity for and hence a selective action on parasitic cells (*nihil agit nisi fixatur*) This selective chemical approach led him to the development of several drugs useful in the treatment of protozoal and spirochetal diseases The best known of these is the organic arsenical arsphenamine (salvarsan) which under the name of 606 reigned supreme in the treatment of syphilis until the introduction of penicillin Ehrlich failed in his attempts to discover the magic bullet which would be so specific for the microbes to be attacked that it would not damage tissue cells nor was he successful in developing drugs active against the true bacterial rickettsial and viral infections Nevertheless there is no doubt that his work constitutes even today the most visionary and rational approach to chemotherapy

The first drug to prove to be effective in the treatment of bacterial infections was the dye Protonsil which was first used by Foerster for the treatment of experimental streptococcal infection and was formally introduced by Domagk (1935) of the I G Farben industry in Germany At the Pasteur Institute in Paris Trefouel soon discovered that the effective agent in Protonsil is not the dye itself (which by the way is completely inactive in vitro) but its simple cleavage product sulfanilamide Thus was opened the chemotherapeutic era and the possibility of synthesizing many derivatives of sulfanilamide clinically superior to the initial product The discovery by Woods in 1940 that sulfanilamide acts by interfering with the utilization of the essential growth factor para aminobenzoic acid led to the theory that metabolic competitive inhibition is the basis of drug action It appeared then and may still be true that the search for antimetabolites constitutes the most rational approach to the discovery of antibacterial drugs In fact

however the greatest therapeutic advances have come from another approach less well defined chemically and indeed almost empirical but more effective in practice

In 1877 Pasteur had observed that cultures of anthrax bacilli often died after becoming contaminated with soil bacteria and he suggested that this might have useful implications for the development of therapeutic agents Many observations have since confirmed that certain microbial products are toxic for various microbial species One of these was made by Alexander Fleming (1881 1955) who noticed in 1929 that an extract of a certain Penicillium had powerful antibacterial properties (see biography by A Maurois 1959) Fleming named the active material penicillin but he could neither purify it nor obtain it in quantities sufficiently large for therapeutic assays In 1939 R Dubos developed a general technic which permitted the systematic search for antimicrobial agents among saprophytic organisms It led to the isolation from a soil bacterium of an antibacterial agent tyrothricin which was active in the treatment of experimental infections in mice The active material proved to be a polypeptide (gramicidin) which was crystallized produced commercially and used in the treatment of infected wounds and of bovine mastitis however its toxicity precluded its use in systemic infections In 1940 H W Florey and E Chain succeeded in preparing Fleming s penicillin in a stable form crystallized it and demonstrated its phenomenal effectiveness in vivo as well as its lack of toxicity Thus began a new era in antibacterial therapy which was marked by the discovery in rapid succession of several highly effective drugs obtained from molds and actinomycetes Streptomycin discovered by S A Waksman in 1944 deserves a special mention here because it proved to be highly effective in the treatment of tuberculosis

The enthusiasm created by the discovery of sulfanilamide penicillin and other antibacterial drugs was so great that many physicians and scientists were led to believe that the conquest of infectious disease had finally been achieved Soon however it became evident that several fundamental and probably inescapable difficulties stand in the way of eradication of disease by drugs Most microbes readily develop mutant forms which are drug resistant and it is proving difficult to discover new drugs fast enough to cope with this situation Even more important *however is the fact that many microbial* forms persist in a dormant state in the tissues even after successful therapy The problem of the persisters is as disturbing for the student of chemotherapy as the existence of the carrier state is for the student of epidemiology



Fig 9 Sir Alexander Fleming (1881 1955) (Vogue 1953 Conde Nast Publications)

THE EVOLUTION OF EPIDEMIOLOGIC THOUGHT

All ancient leaders of roaming tribes and conquering armies had to enact sanitary regulations in order to check the spread of

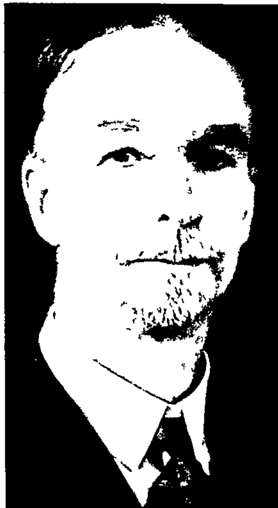


FIG 10 Theobald Smith (1859 1934)

filth diseases Thus Moses formulated strict regulations for camp life before leading his people across the desert In the words of the medical historian Garrison The ancient Hebrews were the founders of prophylaxis and their high priests were true medical police The admonitions in Leviticus the various forms of taboos and quarantines found in all civilizations and the segregation of lepers during the Middle Ages are only a few of the many examples proving that practical men have long realized empirically that many diseases are communicable and that the best protection against them is to prevent their spread

The enormous shifts of people brought about by the Industrial Revolution and the attendant crowding in the filthy tenements of the mushrooming cities created conditions favorable for the spread of infectious diseases during the 19th century The prevalence of epidemics stimulated important epidemiologic studies which led to highly effective control practices even before the pathogenic role of microbes had been convincingly established The few classic examples now to be mentioned should be considered only as illustrations of the many facts which were established during the premicrobial era

In 1854 the cholera epidemic in London provided John Snow (1813 58) with the opportunity for a spectacular demonstration that water constitutes the vehicle through which this disease is transmitted Within 250 yards from the spot in Broad Street where the outbreak began there were 500 deaths from cholera in 10 days With unerring exactitude Snow traced the outbreak to a particular pump in Broad Street and found evidence that its water was contaminated with organic matter Even though he knew nothing of cholera vibrios he thus demonstrated that cholera was water borne and he stopped the epidemic so the story goes by removing the handle from the pump William Budd (1811 80) also provided convincing evidence that typhoid is transmitted by the excreta of the patient again without the benefit of bacteriologic knowledge In Munich Max von Pettenkofer (1818 1901) went far toward ridding his city of typhoid fever simply by cleaning it up and providing it with pure water brought directly from the mountains And similarly Florence Nightingale (1820 1910) effected her reforms of hospital sanitation simply through common sense practices and through her belief in pure air pure water and cleanliness

Florence Nightingale as well as von Pettenkofer remained to the end indifferent and even hostile to the germ theory of disease therefore it is of particular interest that they were so highly effective in combating infection Countless similar examples could be quoted to illustrate that shrewd epidemiologic observations and the development of valuable sanitary practices are not entirely dependent on bacteriologic knowledge The control of malaria and of yellow fever

through environmental changes which eliminated mosquitoes constitutes other examples of medical achievements based on epidemiologic rather than etiologic understanding

Needless to say epidemiologic analysis became much easier and more precise when it could be based on knowledge of causative agents One needs only mention as example the demonstration by A Gartner in 1888 that an outbreak of food poisoning in a human population had been caused by *Salmonella enteritidis* which had contaminated the meat of a sick cow—a finding which provided the pattern for the study of human disease a sociated with animal infections The control of water borne typhoid became much easier and more dependable after Theobald Smith (1859 1934) had shown in 1892 that the enumeration of coliforms in water provides a useful index of fecal contamination Worth mentioning also is the demonstration by Theobald Smith and J Ravenel that bovine tuberculosis can be transmitted to human beings for example through contaminated milk A very large chapter could be written on the enormous practical importance of the demonstration that many protozoan bacterial rickettsial and viral diseases are transmitted by insects Plague rodents and their fleas have played a large part in the history of mankind and their interrelationships became clear only in the early years of the 20th century Hans Zinsser made typhus the topic of a best seller as well as of a fascinating account of human epidemiology in his famous books *Rats Lice and History*

Progressively the knowledge concerning microbes their vectors of transmission the factors which affect their spread and the susceptibility of human populations to them became so extensive that epidemiologists found it possible to describe the course of epidemics in mathematical terms In several cases indeed the shape of the epidemic curves proved to be almost predictable for each type of infection Epidemiology thus progressed from qualitative and descriptive knowledge to the status of a quantitative science However it is becoming apparent that the greatest difficulties in the understanding of epidemics and in the prediction of their course come not so much from a *quantitative* as from a *qualitative* deficiency of knowledge Some of these deficiencies deserve mention here because they point to areas of ignorance which must be explored before further progress can be made in understanding the determinants of infection and of epidemics

Direct transmission from patient to patient or indirectly through insect vectors water food and fomites accounts for only part of the spread of disease One of the most obscure and important aspects of epidemiology is the persistence in healthy persons of pathogens which can initiate disease processes under ill defined conditions The persistence of diphtheria bacilli in patients who had completely recovered was demonstrated as early as 1890 by E Roux A Yersin and F Loeffler And ever since the existence of carriers has been recognized in practically every type of infection Typhoid Mary is only the symbol of a type of latent infection which occurs not only in typhoid fever but also in countless other infectious diseases as well Furthermore the problem has become still more complicated during recent years by the discovery that persisters commonly resist even the most vigorous forms of chemotherapy It is now realized that many potential pathogens are widely distributed through the community yet they give rise to overt disease only under special conditions As a result of this awareness there is developing an increasing preoccupation with the factors which convert infection into disease In other words interest in the properties of the microbial agents of disease is being supplemented by a lively concern with the genetic hormonal metabolic and *nutritional factors of the host which affect* his resistance to infection The essays by Theobald Smith (1934) and N H Swellengrebel (1939) are representative of this change of emphasis

The significance attached to the expression crowd diseases illustrates well the shift of emphasis from the microbe itself to the host response Until recent years students of infection had only a one sided view of crowd diseases They took it for granted that crowding brings about an increase in infectious disease simply because it facilitates the transmission of pathogens from one per on to the

other However observations on animals and man have now revealed that crowding profoundly affects physiologic processes even in the absence of infection for example it increases the activity of certain hormonal systems and disturbs certain metabolic functions In consequence crowding can increase the prevalence and the severity of infection not only by facilitating the transmission of pathogens but also by decreasing host resistance to them

RETROSPECTIVE AND PROSPECTIVE

The history of medical microbiology thus provides a striking support for the view that the development of science occurs according to a dialectic pattern Until the 19th century the medical scene was dominated by the Hippocratic doctrine infectious disease was regarded primarily as the consequence of some failure in the host After 1880 it appeared that all infectious processes could be accounted for by purely microbiologic explanations—in terms of types numbers properties and structures of the invading pathogens Today attempts are being made to integrate these two attitudes into an ecologic doctrine which would be the synthesis of the physiologic thesis and the microbial antithesis

In fact the ecologic doctrine is making it clear that the problem of infectious disease involves more than the interplay between the pathogen and the individual host Epidemics are phenomena which cannot be understood only from the happenings in individual patients they must be studied at the population level The response of a group to infection exhibits characteristics which are less the expression of the individual members of the group than of the interplay between them In most cases for example the course of tuberculosis can be understood only by regarding the disease as a social phenomenon The past and present history of the group determines the manner of its response to the tubercle bacillus and tuberculous infection in turn affects the behavior of the group as a whole (Dubos and Dubos 1952)

Man does not and could not live in a biologic vacuum Microorganisms are an inevitable part of his environment His response to them is conditioned by his ways of life and on the other hand the effects of microbes on him are so varied and so great that they affect the structure of his societies To be fully comprehended medical microbiology must be considered as an ecologic science

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2

The Evolution of Microbial Diseases

THE SO CALLED CONQUEST OF MICROBIAL DISEASES

By the 1950 s the most optimistic dreams of the founders of medical microbiology had been essentially fulfilled in several countries of Western civilization A very large percentage of the microbial agents of disease had been isolated identified and cultivated in artificial media or in tissue cultures bacteriologic purity of the food and water supplies had become possible through technologic advances practical procedures had been worked out for the large scale production of killed or attenuated vaccines highly effective drugs had become available for the treatment of bacterial and parasitic infections a variety of pesticides had been synthesized and had proved their usefulness for the control of insect vectors

In many places economic prosperity and social organization have now made it possible to take full advantage of the extraordinary scientific achievements of the microbiologic era As a result the mortality rates of infectious diseases have been brought down to a very low level particularly among children and young adults As a result the life expectancy at birth has soared to unprecedented high levels

Therefore most clinicians public health officers epidemiologists and microbiologists felt justified in proclaiming during the 1950 s that the conquest of infectious diseases had finally been achieved Surprising enough this euphoria has not yet been dampened by the increasing awareness that the morbidity rates of infection have not decreased significantly and in some cases have actually increased Despite so much oratory on the conquest of microbial diseases the paradox is that the percentage of hospital beds occupied by patients suffering from infection is now as high as it was 50 years ago Today as in the past disorders of the respiratory and the digestive tracts with a microbial etiology constitute the most frequent causes of absenteeism from school office factory or from training in the Armed Forces

There is a widespread tendency to explain away the fact that infection remains a major cause of disease by invoking the emergence of drug resistant forms of bacteria the appearance of new strains of pathogenic agents and the failure to apply with sufficient vigor the existing knowledge of prophylaxis and therapy In reality however these explanations deal with only minor aspects of the problems The more important reason for the stubborn persistence of infection lies in obscure phenomena concerning the interrelationships between man and his biologic environment As we shall see there are important aspects of the infectious problem which are not controlled by sanitation vaccines or drugs (Dubos 1965)

The point at issue is that the microbial diseases which account for the greatest morbidity in our communities today are completely different in their origin and manifestations from those which are dealt with so effectively by modern technics For this rea

son they will require programs of study and of control different from those which have been emphasized so far The real problem is not how to apply more effectively the control procedures that we already possess or how to improve them but rather to search for a qualitatively different kind of knowledge

The sciences concerned with microbial diseases have developed almost exclusively from the study of acute or semiacute infectious processes caused by virulent microorganisms acquired through exposure to an exogenous source of infection In contrast the microbial diseases most common in our communities today arise from the activities of microorganisms which are ubiquitous in the environment which persist in the body without causing any obvious harm under ordinary circumstances and exert pathologic effects only when the infected person is under conditions of physiologic stress In such a type of microbial disease the event of infection is of less importance than the hidden manifestations of the smoldering infectious process and than the physiologic disturbances which convert latent infection into overt symptoms and pathology This is the reason why the orthodox methods based on the classic doctrines of epidemiology immunology and chemotherapy are not sufficient to deal with the problem of endogenous diseases The need is to develop procedures for re establishing the state of equilibrium between host and parasite

The problems posed by microbial diseases of endogenous origin cannot be stated properly without some historical considerations of the changes in virulence that infectious agents have undergone in the course of time From this point of view as we shall see it is not very enlightening to say of a particular microorganism that it has a high or a low virulence A more meaningful statement is that a given pathogen is generally highly destructive in a given population when the two components of the system first come into contact and that the severity of the infectious process tends to decrease as contact between population and pathogen is continued over several generations In other words the problem of virulence cannot be stated without historical perspective

HISTORICAL CHANGES IN INCIDENCE AND SEVERITY OF VARIOUS MICROBIAL DISEASES

History provides many examples of the fact that social misery and its usual consequence physiologic misery is commonly associated with increase in the prevalence and the severity of microbial diseases War famine and pestilence have been known to ride together throughout history

In many cases of course the infectious diseases associated with crowding or war are directly the result of increased contact with particular types of microbial agents Armies in the field have been plagued by typhoid and dysentery whenever the breakdown of sanitary practices has permitted massive infection with salmonellae and shigellae Napoleon s troops contracted typhus when they were heavily exposed to lice and rickettsia in Eastern Europe during the 1812 Russian campaign Similarly the Western allies suffered much from scrub typhus and malaria in the Asiatic theater during World War II even though the adult native populations with whom they were in contact suffered much less from these diseases to which they had been exposed throughout life

Of equal importance and greater interest perhaps is the fact that crowding wars and social upheavals can increase the prevalence of microbial disease through other mechanisms namely by creating conditions which decrease the general resistance of the body

The tuberculosis epidemic which prevailed throughout the Western World during the 19th century owed part at least of its severity to the long working hours the poor nutrition and the low living standards prevailing among the labor classes during the Industrial Revolution As living standards improved tuberculosis mortality immediately began to decrease The improvement was already noticeable at the end of the century long before any specific measure of prophylaxis or therapy had been introduced Tuberculosis mortality again exhibited a sharp and sudden rise in parts of Europe during World Wars I and II and once more it resumed its downward course as soon as the hostilities were over

During the 1920 s inflation in Germany

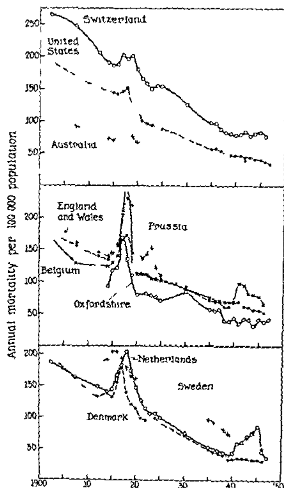


Fig 1 The effect of war on tuberculosis The figures at the bottom indicate the years those at the left the number of deaths per year per 100 000 population (Dubos & Dubos 1952)

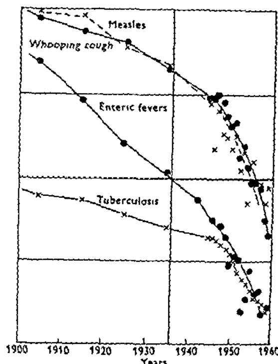


Fig 2 Shows the trend of mortality from some infectious diseases in England and Wales during the 20th century Relative death rates are shown for measles whooping cough enteric fevers and tuberculosis The absolute scales differ but all are shown logarithmically the horizontal lines indicating a 10 fold difference Between 1936 and 1960 there has been almost a *100 fold* diminution in the deaths from whooping cough and measles (Burnet F M Natural History of Infectious Disease ed 3 Cambridge University Press 1962)

provided a spectacular illustration of the bearing of social factors on resistance to disease Following an abrupt fall at the end of the war the tuberculosis mortality increased sharply during the years of inflation and fell again as soon as stabilization of the currency allowed a return to more normal living conditions The rapid and reversible *manner in* which the social body responds to changes *in the social structure* by changes in tuberculosis mortality rates cannot be explained in terms of infection rates The more likely explanation is that large numbers of persons in our communities are in a state of unsteady equilibrium with tubercle *bacilli as well as with many other pathogens and that this* equilibrium is upset whenever disturbances impair the general state of resistance of the body (Dubos and Dubos 1952)

This relationship has been brought out in an analysis of the disease problems in German concentration camps during World War II *Among infectious diseases it was not the* exotic unusual epidemics like typhus cholera or even bacillary dysentery which proved to be the most troublesome in the camps but rather ordinary skin ailments colds bronchopneumonias staphylococcal infections pulmonary tuberculosis—in other *words the type of diseases minor or severe*

caused by microorganisms *endemic* in the normal European communities (Larsen *et al* 1952) Here again increase in con tact infections could hardly account for the aggravation of these endemic diseases Far more important certainly was the loss of natural resistance caused by malnutrition and other forms of physiologic misery It was remarkable indeed that most of the internees overcame their microbial maladies shortly after their return to a normal environment often without the help of specific therapy Even in the case of tuberculosis rapid re covery was the rule though no antimyco bacterial agent was available for treatment of the disease until the late 1940 s

The spectacular decrease in the mortality caused by certain microbial diseases in our communities is due in part to methods of prevention and therapy but in part only Im portant though it has been the effect of these methods does not explain the changes which have occurred during the past century The spectacular health improvements which have recently occurred in Puerto Rico illustrate well that the evaluation of the role played by control programs is complicated by a number of unresolved epidemiologic puzzles

As late as 1950 diarrhea and enteritis were listed as the leading causes of death in Puerto Rico with a mortality rate of 138 for 100 000 population This rate had fallen to 33 in 1962[1] The reasons for this startling change are obscure since the fundamental cause of most intestinal disorders is still today a matter for speculation and since neither antimicrobial vaccines nor drugs could have been used for prophylaxis or treatment During the same period the mor tality from measles whooping cough and scarlet fever has also been reduced drasti cally again without the benefit of specific methods of prophylaxis or treatment

These epidemiologic puzzles are not peculiar to Puerto Rico Thus the mortality from tuberculosis from various diseases of the respiratory and the digestive tracts and especially from viral infections began to fall precipitously in Europe and in the U S A long before the development let alone the use of specific methods of control (Dubos and Dubos 1952 Burnet 1962) (Figs 1 and 2) Granted that the hereditary levels of innate resistance and susceptibility may differ from one national group to another the evolution of the microbial diseases of man strongly suggest that these character istics are not necessarily of racial origin Usually they are rather the consequence of the extent and the duration of the contact that a particular group of people has had with a particular microbial agent The history of disease among the various Indian and Polynesian populations is instructive in this regard

Smallpox is thought to have been intro duced into the American continent early during the Spanish Conquest probably by a Negro in Cortez band The Indians proved to be highly susceptible to the disease which almo t wiped out some of their settlements and there is reason to believe that this dis aster contributed to their rapid defeat by the Spaniards The conquest of North America a century later provided further dramatic evidence of the susceptibility of the Amer indians to smallpox Repeated outbreaks decimated village after village and at times whole tribes Early in the 17th century for example the Massachusetts and the Narra gansett Indians were reduced from presmall pox populations of 30 000 and 9 000 re spectively to less than 1 000 in a few years In the epidemic of 1837 the Mandan popu lation fell from 1 600 to 30 the Assiniboins lost whole villages the Crows one third of their population while the total deaths among the Plains tribes amounted to 10 000 in a few weeks Similar outbreaks occurred as late as 1870 71 among the Blackfeet

Other microbial diseases brought in by the European invaders contributed still further to the rapid decline of the Indian populations during the 19th century There were more than 700 000 Indians in North America before the arrival of the Europeans but only 250 000 in 1850 Tuberculosis played a large part in this holocaust as illustrated by the epidemics which decimated the Plains Indians of Western Canada In the 1890 s the death rate from tuberculosis in the Qu Appelle Valley reservation of Sas katchewan reached the fantastic figure of 9 000 per 100 000 population per year[1] More than half the families of this tribe were eliminated in the first 3 generations of the

epidemic Some 20 per cent of the deaths in the surviving families were caused by tuberculosis! (Ferguson 1955)

Microbial diseases among the Polynesians during the past century present a picture similar to that seen among the Amerindians The South Pacific was explored during the second half of the 18th century Granted that the charm of the Pacific Islands and the amorous welcome of their women may have warped the judgment of the European visitors there is no reason to doubt the validity of their unanimous opinion concerning the health and the vigor of the Polynesian people at that time The Europeans saw throughout the Islands robust and happy men and women obviously well adapted to their physical environment as well as to their local pathogens Yet microbial diseases became rampant among the South Sea islanders within a short time after these early explorations and the Polynesian population soon began to fall From approximately 300 000 at the time of Captain Cook s first visit in 1778 the population of native Hawaiians had fallen to 37 000 in 1860 During the same period the population of the New Hebrides was reduced to one tenth of its original size

There is no doubt that this catastrophe was brought about largely by venereal diseases tuberculosis scarlet fever measles and other infectious disorders that the Polynesians contracted during their short contacts with the Europeans When measles was introduced into Hawaii practically the whole population went down with the disease and many thousands died Epidemics of measles pertussis and influenza occurred again in 1848 and every child born that year died When smallpox struck in 1853 there were over 9 000 cases with 6 000 deaths out of a population of 70 000 During his second and third visits to the South Pacific Captain Cook himself was much disturbed at the thought that his sailors were responsible for having introduced venereal diseases among the Polynesians in Tahiti However he found solace in the belief that the initial guilt was to be placed on Bougainville s French crew who had preceded them

Many other epidemic outbreaks of disease could be selected to illustrate the destructiveness which viruses bacteria and protozoa often exhibit when first introduced into a population In Europe, plague caused a fantastic mortality during the Justinian era and again during the Renaissance The sweating sickness that suddenly struck certain areas of England in Tudor times also proved to be disastrous as can be judged from Caius classic treatise A Boke or Counseill Against the Disease Commonly Called the Sweate or Sweatying Sicknesse

The fact that a disease commonly exhibits extreme virulence when newly introduced into a population was once more illustrated in a dramatic manner by the outbreak of measles in 1952 among the Eskimos of the Canadian Arctic The attack rate in this instance was over 99 per cent, including all ages and the mortality rate reached up to 7 per cent at Ungava Bay An outbreak of poliomyelitis among a group of Eskimos of the Hudson Bay in 1949 exhibited the same pattern Fourteen per cent of the population died, and over 40 per cent developed paralytic disease In this case again all age groups were involved the lowest clinical attack rate being in the infants

Still another example made familiar by the writings of Albert Schweitzer is provided by African trypanosomiasis also known as sleeping sickness Trypanosomiasis was introduced in the Ogowe region of Equatorial Africa some 40 years ago by carriers that came with the Europeans from Loango where it apparently has existed from time immemorial The disease proved to be terribly destructive in its new territory carrying off one third of the population in the course of a few years In Uganda it killed 200 000 persons out of 300 000 in 6 years Of the 2 000 inhabitants in a village of the Upper Ogowe only 500 survived after 2 years of the epidemic

The examples cited above—and these are only a few among many others that would tell the same story—leave no doubt that whole populations can be literally decimated by pathogens with which they have had little contact in the past It is apparent on the other hand that the diseases introduced by the white man among the Indians the Polynesians and other primitive people during the 18th and the 19th centuries no longer

affect them as acutely and with as rapid a fatal outcome as was uniformly the case in the past These people have developed or are developing biologic responses to infection which are similar to those seen in the Western world under normal conditions

The high mortality rates caused by the plague bacillus or by the yellow fever virus among Europeans probably can serve as an analogy for the degree of virulence that the tubercle bacillus or the measles virus had for the Polynesians or the Indians 2 centuries ago Tuberculosis and measles are still important causes of disease among these people today, but as already mentioned they rarely give rise to catastrophic epidemics in the areas where they have been established for several generations Similarly it has been observed that after a while African trypanosomiasis loses some of the destructive character that it exhibited when first introduced into new districts of Equatorial Africa The disease lingers on but it carries off small numbers of victims instead of killing two thirds of the exposed populations as it once did

Precise observations are available concerning the changes in the clinical manifestations of tuberculosis among some Indian tribes of North America In the first and the second generations to suffer from the tuberculosis epidemic in the Qu Appelle Valley reservation extensive glandular involvement was the rule in school age children Meningitis generalized miliary disease and bone and joint disease were extremely frequent—evidence of inability of the host to localize infection In 1921 at a time when the generalized epidemic was in the third generation the disease showed a greater tendency to localize in the lung and to exhibit a chronic course the mortality was falling and glandular involvement had dropped to 7 per cent among school children This latter manifestation of high susceptibility to tuberculosis has continued to decline steadily and was seen in less than 1 per cent of children in the 4th generation In other words while tuberculosis among the Amerindians exhibited at first a very acute course different in character from that observed in people who have had contact with the tubercle bacillus for several generations now it is undergoing a change which makes it resemble the more chronic type of disease commonly seen among Western people under normal conditions (Ferguson 1955)

EXOGENOUS VERSUS ENDOGENOUS MICROBIAL DISEASE

Irrespective of the specific nature of their etiologic agents and of their pathologic manifestations microbial diseases can have two different kinds of origin They can be either exogenous or endogenous

Many pathologic processes are of course the direct outcome of exposure to a virulent pathogen Smallpox yellow fever typhus syphilis diphtheria tuberculosis typhoid fever malaria etc illustrate situations in which the disease can usually be traced to a well-defined exposure to the responsible microbial agent the pathologic phenomena then usually develop within a fairly predictable period of time after exposure This truly *infectious* kind of microbial disease with an *exogenous* origin is still of immense importance throughout the world but it has become less of a menace in our own communities thanks to public health practices vaccination prompt diagnosis and antimicrobial therapy

In contrast the microbial diseases which are now gaining prominence especially in the prosperous countries of the Western world are often caused by microbes formerly regarded as essentially harmless for example the coliform and other gram negative bacilli the nonhemolytic streptococci various kinds of yeasts as well as fungi and probably many viruses still unidentified Even though their virulence is low nevertheless these organisms can give rise to serious pathologic states under special physiologic circumstances

The expression *endogenous* microbial disease refers to any pathologic state caused by microorganisms acquired at some prior time which have persisted in the body as part of its indigenous microbiota (see Chap 14) However it is important to emphasize that highly virulent pathogens such as tubercle bacilli can also act as etiologic agents of endogenous disease The reason is that most types of virulent organisms if not all can become established in the body and persist

in it without manifesting their presence in the form of overt disease until the general resistance of the host is lowered

Endogenous microbial diseases have always been responsible for a very large amount of human misery but two developments have increased their relative importance and made them more obvious during recent years One is that the exogenous infectious processes are increasingly being brought under control a situation which has made it easier to recognize the microbial diseases of endogenous origin The other development is that paradoxically enough many therapeutic procedures introduced by modern scientific medicine favor the proliferation of certain components of the indigenous flora and thus allow them to cause disease For example enteritis caused by staphylococci yeasts or fungi frequently occurs when the normal intestinal ecology is disturbed by the administration of antimicrobial drugs Similarly the microorganisms of the indigenous flora often proliferate after treatment with corti one or antileukemic drugs as well as following certain surgical procedures such as subtotal gastrectomy

Awareness of the fact that most pathogens are widely distributed and yet do not cause clinical disease except in a very small percentage of persons and animals harboring them has come chiefly from the study of latent viral and rickettsial infections However latent infections with virulent bacteria fungi and higher parasites are in fact at least as common as are those with viruses and rickettsia Surveys made in different parts of the world show for example that 30 to 50 per cent of normal persons harbor coagulase positive staphylococci in their nasopharynx

Until a few years ago practically all human beings in the Western world did become infected with tubercle bacilli and this situation still exists today in most underprivileged countries yet only a small percentage of tuberculin positive persons develop clinical tuberculosis In the United States *Amoeba histolytica* can be isolated from the stools of a surprisingly large number of persons who have never suffered from amebiasis Similarly some 30 per cent of the population give a positive skin test for toxoplasmosis and encysted trichina are often found in muscles in the absence of any clinical sign of trichinosis Indeed the list of latent infections with all classes of pathogens continues to grow as the search for them is expanded

A few examples taken from the fields of animal and human pathology will illustrate how endogenous diseases manifest themselves under natural conditions

Shipping fever also known as transit fever is a severe disease which strikes farm animals when they are moved from one area to another under conditions of stress the disease is caused by a great variety of microbial agents Calves, for example commonly develop pneumonia or enteritis during transit and pigs become susceptible to the pathologic effects of *Erysipelothrix* which they carry in a latent state (Francis 1959) Interestingly enough, most of the manifestations of shipping fever can be prevented by treating the animals with tranquilizers at the time of shipment In some unexplained manner these drugs minimize the physiologic disturbances which activate the indigenous microbial agents involved in shipping fever

Two surveys recently made in large American hospitals highlight the importance of endogenous microbial diseases in man At the New York Hospital 54 per cent of the fatal infections observed in 1957 58 occurred while the patients were under care on the medical wards most of the infections were caused by microbes of low virulence and were brought about by therapeutic procedures directed at other disease conditions (Rogers 1959) At the Mayo Clinic only half of the 294 cases of meningitis studied during the 1950s could be traced to the classic etiologic agents of this condition namely meningococci pneumococci or influenza bacilli the other cases were caused by common organisms such as *E coli Pseudomonas* coagulase negative staphylococci etc not usually regarded as virulent

MICROBIAL PERSISTERS

The fact that most pathogens—viruses rickettsia fungi bacteria protozoa and even helminths—can persist in the body in a non active state for prolonged periods of time

and that many types of microbes are always present in the tissues of all normal individuals is at the origin of some of the most important and most perplexing problems of epidemiology and clinical medicine Furthermore it has given rise to an extensive and rather confusing terminology The expressions carrier state latent inapparent or dormant infection masking and unmasking of viruses etc have never been clearly defined and it is often difficult to differentiate the shades of meaning which they are supposed to convey More recently the word persister has been introduced to designate the parasites which survive in tissues despite successful and prolonged chemotherapy (McDermott 1958 1959)

It will suffice to point out here that pathogens and other microbes can persist in the body in a number of different states At one extreme are those situations in which the pathogen exists in the tissues in a form which can be demonstrated readily by standard technical procedures The recovery of typhoid bacilli from typhoid carriers or of the virus of herpes simplex from the tissues of normal persons are examples in point At the other extreme are the situations in which recovery of the pathogen is all but impossible as long as the latent infection has not evolved into an active pathologic process in the infected animal or person For example the epidemic strain of typhus (*Rickettsia prowazeki*) can persist in man for many decades and remain undetected until it causes the Brill Zinsser disease or again murine corynebacteria can persist in an undetectable form in rodents yet multiply explosively as a result of stress nutritional deficiencies or treatment with massive doses of cortisone (Fauve *et al* 1964)

Technical difficulties often account for the failure to isolate the pathogen Indeed it is only in specialized cases that the methods in use at present permit detection of a single or a few infective units Therefore the report that a pathogen is inapparent or masked must always be judged in terms of the sensitiveness of the procedure used to test for its presence In many cases infected tissues contain growth inhibitory substances which although not capable of destroying the pathogen which is being looked for in vivo nevertheless can inhibit its multiplication in vitro For example neutralizing antibody transferred from infected tissue along with a virus is likely to prevent the latter from becoming established in a recipient animal Tissue inhibitors other than antibodies can also cause difficulties as when tissue constituents prevent the growth of tubercle bacilli in otherwise favorable culture media Elimination of neutralizing antibodies and of other inhibitory agents is therefore a prerequisite for the successful demonstration of the presence of small numbers of infective units in the tissues but few if any are the cases in which this can be done adequately

Even more subtle mechanisms may be involved in the failure to recover pathogens from infected tissues Viruses can persist in vivo in the form of naked nucleic acid structures and bacteria in the form of protoplasts as long as they are sheltered in the well balanced environment of their hosts However such delicate infective structures are readily destroyed or inactivated when removed from their protective environment and as a result they usually escape detection by the customary laboratory procedures The role of such naked infective particles in disease has not yet been fully documented but their very existence illustrates nevertheless that many different mechanisms can account for the well established fact that microbes often persist in the tissues even when it is impossible to demonstrate their presence by ordinary microscopic or cultural technics Finally microbes can persist in a form which though readily culturable is so atypical as to prevent recognition For example the agent of murine corynebacterial pseudotuberculosis persists in vivo as an avirulent variant which reacquires virulence and multiplies extensively when the rodent is treated with cortisone (Fauve *et al* 1964)

Oddly enough the phenomenon of persistence has become more obvious and perhaps of greater practical importance as a result of the widespread use of chemotherapeutic agents Whereas antimicrobial drugs are often highly successful in arresting the progress of disease they rarely succeed in eradicating the causative agent from the tissues For example even after successful

treatment of scrub fever with tetracycline of gonorrhea with penicillin or of tuberculosis with isoniazid and other antimycobacterial drugs some of the causative rickettsia gonococci or tubercle bacilli persist in the tissues (McDermott 1958 1959 Smadel 1963) And so it is with many other types of infectious agents even following treatment with highly effective drugs

It is important to emphasize that contrary to common belief persisters are not necessarily forms of the organisms which have become genetically resistant to the drug used for therapy More often than not they are still drug susceptible in the genetic sense *but either they exist in a physiologically* resting form on which the drug is not active or they are located in parts of the body or in tissue cells which provide a protective shelter for them (McDermott 1958, 1959)

The production of persisters is not a phenomenon peculiar to chemotherapy It occurs also in the presence of protective antibodies because immunologic processes are rarely if ever more successful than antimicrobial drugs in fulfilling Paul Ehrlich s ideal of absolute sterilization In other words viruses bacteria and other pathogens can persist in the tissues even when the level of specific immunity *is high enough* to prevent the infection from progressing

THE PHENOMENON OF INFECTION IMMUNITY (PREMUNITION)

It has long been recognized that the persistence in the body of a given microbial agent is accompanied by a high level of resistance to superinfection with this same agent Such a state of resistance was early recognized and designated as infection immunity or premunition Although emphasized chiefly for its relevance to malaria tuberculosis syphilis and relapsing fever infection immunity is certainly of very general occurrence but its study has been grossly neglected New observations made during recent years should renew and stimulate scientific interest in this unorthodox yet highly interesting field of theoretical and practical immunology

An illuminating example of infection immunity has been provided recently by the study of the comparative resistance of several strains of mice to corynebacterial pseudo tuberculosis Mice of the C57 Black strain are highly resistant to the causative agent of this disease *Corynebacterium kutscheri* whereas mice of the albino Swiss strain are highly susceptible Cross mating between the 2 mouse strains gives results suggesting that their difference in susceptibility has a genetic basis However when normal C57 Black mice are treated with large doses of cortisone they succumb from spontaneous endogenous infection with *Corynebacterium kutscheri* a fact which proves that they normally harbor this organism In contrast, albino Swiss mice subjected to the same treatment do not develop the endogenous disease and therefore must not be carriers This correlation between carrier state and resistance to superinfection has been established for many other strains of mice In other words resistance to superinfection with the corynebacterium is in some manner correlated with the presence of persisters of this bacterial species (Fauve *et al* 1964)

These findings bring to mind the response of experimental animals to certain viral infections For example the virus of lymphocytic choriomeningitis produced a severe disease with *fatal outcome* when it was first introduced among albino mice but eventually it became established in several mouse colonies Within a few years after its introduction it was found to be present in all animals of these colonies producing in them a latent infection The infection was contracted in utero but did not manifest itself by any detectable sign of disease in adult animals even though their organs continued to harbor active virus throughout life In this case therefore a few years sufficed to change the type of relationship between mice and choriomeningitis virus from that of a virulent epidemic to a state of silent commensalism or symbiosis In many colonies the virus has become an unobtrusive guest the presence of which is barely noticed by the host but its presence renders the carrier mice resistant to superinfection

The response of animals to the polyoma virus provides another striking illustration of the resistance afforded by contact early in life with an infectious agent Injection of

the virus into a variety of species of experimental animals is likely to result in the production of many different kinds of tumors However it seems that this happens only if the animals used are obtained from a colony free of polyoma In contrast there is no evidence of tumor production in wild or laboratory populations of animals which normally carry the virus Therefore as in the case of corynebacteria the acquisition of a viral agent either in utero or very early in life may fail to produce any sign of disease yet profoundly alter the response of the host that harbors it to subsequent infection with this virus (Huebner 1961)

The findings just outlined point to the paradoxical conclusion that animals and probably human beings may behave as if they were genetically resistant to a given agent yet owe this behavior in reality to some modification of their tissue response brought about by the fact that they carry persister forms of this very agent Such infection immunity or premunition may also play some part in the life long immunity to viral infections which follows early exposure to the pathogen or vaccination with attenuated living vaccines

It is certain that the conditioning of response brought about by infection in utero or very early in life operates through several unrelated processes ranging from immune tolerance through cellular and humoral immunity to nonspecific stimulation of defense processes However whatever the theoretical basis of their individual mechanisms the various forms of infection immunity have in common certain practical consequences which suggest possible approaches to the control of some problems of infection It has been observed for example that human beings who carry *Escherichia coli* do not readily become superinfected with other strains of this bacterial species Furthermore recent clinical studies reveal that the colonization of human infants with virulent staphylococci can be prevented or retarded by early contamination nasal or umbilical with very small inocula of a coagulase positive strain of staphylococcus (selected because of its great susceptibility to penicillin and low virulence) Not only does the attenuated strain become lastingly established in the artificially contaminated infants it also spreads to other human contacts and the over all effect is to limit the dissemination of more virulent staphylococci (Shinefield *et al* 1963 Dubos 1963)

Thus it is apparent that the ubiquitous presence of a potential pathogen in a community can confer a high level of resistance to this particular agent Infection immunity probably does not constitute an immunologic entity but through the various mechanisms which permit microbial persistence it may play a very important role in the phenomenon of herd immunity

GENETIC DETERMINANTS OF MICROBIAL DISEASE

The changes which have occurred during historical times in the relation between man and the various microbial agents of disease suggest that either the latter undergo fluctuations in their infectivity and pathogenicity or that the resistance of man can fluctuate It is probable in fact that both mechanisms can operate—in certain cases singly and in others jointly

Of course viruses bacteria and other parasitic agents can undergo mutations affecting most of their characteristics including their immunologic specificity and their virulence While it is legitimate to assume that such mutations occur in nature so far it has not been possible to demonstrate convincingly that they play an important role in modifying the course of human epidemics However observations made with the virus of rabbit myxomatosis in Australia and Europe have established that genetic changes in virulence can occur under natural epidemic conditions

The European rabbit was introduced in Australia in 1859 As it had no natural enemies in its new habitat it spread rapidly over much of the continent and multiplied enormously The rabbit population eventually reached several billions thus creating a grave threat for the agricultural economy of Australia In 1950 the virus of myxomatosis was introduced in an attempt to control the rabbit population Myxomatosis occurs naturally in the wild rabbits of Brazil but merely in the form of a benign tumor asso

ciated with only a mild and transient viremia The relationship between the Brazilian rabbit and the myxoma virus clearly denotes an ancient association resulting in ecologic equilibrium In contrast European rabbits which had never had any contact with the virus uniformly develop an acute almost always fatal disease when inoculated even with very small doses

The initial outbreaks of myxomatosis immediately after introduction of the virus in Australia were characterized by an enormously high case mortality rate—higher than 99 per cent Within a short time however the case mortality had fallen to 90 per cent in areas where a second spontaneous outbreak had occurred Interestingly enough it turned out that this lower mortality was due in part to a decrease in the virulence of the virus Some details concerning this phenomenon are of interest here because the decrease in virulence was unexpected and probably has general significance for epidemiologic theory

In Australia the virus is transmitted from rabbit to rabbit almost entirely through mosquitoes which act mechanically as flying needles Because the highly virulent strains of virus kill rabbits within a very few days the chance for their transmission through the mosquito vector is rather limited However when attenuated mutant strains of the virus appeared spontaneously they produced a less rapidly fatal disease and the rabbit developed skin lesions of longer duration Thus the less virulent mutant strains had a better chance of being transmitted through mosquito bites and they progressively displaced the original highly virulent strain in the field A few years after the introduction of myxomatosis in Australia viral strains recovered from the field caused a mortality of 90 per cent in laboratory rabbits instead of 99 per cent as had been the case with the strain originally introduced (Fenner 1959)

While the evolution of rabbit myxomatosis in Australia can be explained in part by hereditary changes in the virus there is no doubt that it has been influenced also by hereditary changes in the host As already mentioned the European type of rabbit introduced in Australia is immensely susceptible to myxomatosis a disease with which it had no racial experience until a few years ago Now, however rabbits trapped in areas where the infection has been prevalent for several years exhibit a degree of resistance to the most virulent forms of the virus much higher than that exhibited by rabbits before the beginning of the epizootic or by rabbits carefully and constantly maintained in isolation This increase in resistance has a genetic basis and therefore must result from the selection of mutant animals which had survived the initial outbreak Genetic changes in resistance to infection are of course common among animals In fact it is possible almost at will to select and breed laboratory animals having a high resistance or high susceptibility to one or another type of bacterial or viral infections (Lurie 1941 Webster, 1946)

It seems reasonable to believe but difficult to prove that genetic changes also occur in the resistance of man to his pathogens Since epidemics with a great killing power among young people tend to eliminate a large percentage of those having a high degree of susceptibility the likely outcome is the selective survival of that segment of the population endowed with a higher than average innate resistance Thus genetic resistance progressively accumulates in the affected population The history of family groups strongly suggests that such a selective process resulting in higher herd resistance operated in Europe and America during the 19th century epidemic of tuberculosis as it certainly did when tuberculosis eliminated half the families among the Indians of the Qu Appelle Valley reservation (Ferguson 1955 Puffer 1944 Dubos and Dubos 1952) It is probable indeed that the decrease in the severity of tuberculosis in the countries of Western civilization during the past few decades has been brought about not only by improvements in standards of living and in medical care but also in no small part by genetic changes

There are indications that a similar genetic selection has taken place in the Orient with regard to leprosy Among people living today in the Hawaiian Islands the Chinese the Japanese and other Oriental people originating from areas where *Mycobacterium leprae* has long been ubiquitous seem to suffer from this disease far less commonly

than do the Polynesians who became exposed to it for the first time during recent centuries

A slight loss of virulence by the parasite and some increase in the genetic resistance of the host provide the right conditions for the development of acquired immunity in a large percentage of infected individuals Here again rabbit myxomatosis in Australia constitutes an enlightening model Protective antibodies have been found in the surviving animals trapped in infected areas Therefore it can be surmised that the transmission of maternal antibodies helps the young to withstand infection during the first days of life and thereby allows them to develop their own active immunity

The many interrelated aspects of this problem cannot be discussed here but it seems to be worth pointing out that the genetic and immunologic changes favoring resistance to infection which occur during a generalized epidemic are the almost inevitable outcome of prolonged contact between any parasite and any host For this reason it seems unlikely that living species can ever be completely wiped out by epidemics however virulent the parasite The outcome of evolutionary forces is of necessity a *modus vivendi* according to which the parasite and the host reach some sort of equilibrium which permits the survival of both The concept of successful parasitism so ably formulated by Theobald Smith (1934) and N H Swellengrebel (1940) a generation ago is another expression of this evolutionary equilibrium resulting from prolonged racial contact between host and parasite

Evolutionary adaptation between hosts and their parasites helps to explain why the ability or the failure of a particular microorganism to cause disease cannot be fully explained in terms of the concepts encompassed by the word virulence The need to redefine virulence in terms of host parasite relationships will now be illustrated by consideration of malignant malaria in primitive populations

Plasmodium falciparum possesses attributes which make it highly virulent for most members of the human race Yet this parasite produces only a rather mild disease in the Bush Negroes who live in the area included in former Dutch Guinea While over 90 per cent of these Negroes become infected with the malignant form of plasmodium shortly after birth the infection contributes little if anything to infant mortality among them Most adults if not all harbor the parasite in their blood but the intensity of parasitemia decreases with advancing age

The extent of splenomegaly constitutes further evidence of the mildness of malaria in Bush Negroes Among them the spleen rate declines from a high of over 80 per cent in infants to less than 15 per cent in adults The fact that such a large percentage of adults show parasitemia without splenomegaly illustrates the two complementary determinants of the infectious process On the one hand the parasite is virulent since it can become established multiply and persist in the tissues On the other hand the Bush Negro is resistant to the parasite since he can keep its proliferation under control and since his response to its presence is so mild that the infection usually fails to express itself in overt clinical disease (Swellengrebel 1940)

The probable explanation of these findings is that the Bush Negroes of Dutch Guinea have evolved in constant contact with plasmodia Widespread infection constitutes a selective force favoring the kind of genetic endowment which makes it possible for human hosts and virulent parasites to coexist in a state of biologic equilibrium In the case of African Negroes as is well known possession of the sickle cell gene constitutes one of the attributes of resistance to malaria Needless to say immune responses reinforce the effectiveness of genetic mechanisms when the parasite is ubiquitous in the environment

Similar phenomena of adaptive host parasite relationships certainly occur in all other infectious diseases of mankind On a few occasions during historical times the social and economic circumstances proved to be favorable for the spread of *Pasteurella pestis* among the population of Western Europe and every time the mortality that it caused was enormous During the Justinian era in the Roman world and again during the Renaissance in Italy France England and other parts of Europe whole areas were decimated by the bubonic and pneumonic

forms of plague *Pasteurella pestis* is still widely distributed today in certain parts of Asia but granted that it constitutes an important cause of disease in these populations it does not behave toward them as the terrible scourge which almost depopulated Europe on two occasions Similarly cholera can run a fulminating fatal course in people exposed for the first time to its causative vibrio yet the disease is commonly present as a rather mild annoyance in many bazaars of Asia

Like protozoa and bacteria viruses also tend to approach a state of biologic equilibrium with man Thus, a great variety of respiratory infections which are now extremely common and rather mild in our communities are caused by viruses which probably were at a time responsible for more severe pathologic processes Evolutionary adaptation may have taken place even in the case of paralytic poliomyelitis Until recent times most human beings in our communities became infected with polio viruses but only a very small percentage of the infected persons developed paralytic disease in general the infection expressed itself in the form of symptoms so mild as to be overlooked True enough maternal antibodies did account in large part for the failure to develop disease following infection but it is probable that genetic mechanisms of resistance were also involved

Herpes simplex infection is of special interest in this regard because the virus is potentially capable of causing acute and fatal encephalitis More generally however large numbers of children and young adults become infected without displaying severe pathologic signs and they continue to carry the virus throughout life In our communities herpes infection is known chiefly in the form of fever blisters a self limiting disease which occurs only when the carrier of the virus is under some form of physiologic stress Man has come close to achieving a state of biologic adaptation to the herpes virus as well as to many other viruses which are ubiquitous in his communities

THE ACTIVATION OF ENDOGENOUS MICROBIAL DISEASE

In the late stages of evolutionary adaptation between a given parasite and its host in a given community infection is extremely prevalent but it rarely evolves into overt and fatal disease Thus, given enough time a state of peaceful coexistence can be established between any host and any parasite Microbial persistence not accompanied by acute or destructive pathologic processes is therefore not a laboratory freak not a rare phenomenon engendered by tricky manipulations of antimicrobial drugs or immunologic responses Throughout nature, infection without disease is the rule rather than the exception

However latent infections can become activated by many different kinds of changes either in the host or in the environment For this reason a large and most important aspect of the epidemiology of disease (as contrasted with the epidemiology of infection) has to do with the factors which upset the equilibrium between host and parasite and thereby convert dormant infection into overt disease

Maintenance of health despite persisting infection means of course that the mechanisms of physiologic resistance and the humoral or cellular processes involved in acquired immunity are capable of inhibiting the multiplication of the parasites but not of eradicating them from the tissues However this does not imply that acquired immunity necessarily results in freedom from disease for there are many types of physiologic disturbances which allow the parasite to multiply extensively even if the host is specifically immune to it For example persons who carry the virus of herpes simplex commonly have a high level of neutralizing antibodies for this virus in their serum nevertheless they can experience transient episodes of viral multiplication under the influence of nonspecific stimuli—such as certain types of fever excessive exposure to the sun fatigue menstruation or section of the trigeminal nerve The result is then the production of herpes blisters even in the presence of humoral immunity to the virus

On the basis of clinical experience as well as of common observation it is usually considered to be self evident that susceptibility to all sorts of infectious agents is much increased by the various stresses and strains of life Patients as well as their physicians tend to incriminate poor constitution bad

weather lack of sleep or emotional disturbances in the causation of disease even when it is certain that the signs and symptoms result from the activities of viruses or bacteria The life situations which have been assumed to be responsible for the activation of endogenous microbial disease range all the way from the physiologic misery and d privations of internees in concentration camps to the emotional upsets resulting from business failure or an unhappy love affair

Unfortunately few are the cases in which a convincing correlation has been established between the physiologic state of the host and his susceptibility to infection Diabetes constitutes probably the best-documented example of this type of relationship since there is no doubt that the poorly controlled diabetic patient is easy prey to staphylococci tubercle bacilli and many other kinds of bacteria It is also true on the other hand that his resistance to infection becomes almost normal once the diabetes is controlled by adequate insulin therapy In this case therefore the response of the host to infection is under the influence of events perhaps biochemical in nature which can be altered reversibly by the physiologic control mediated through insulin Certain therapeutic procedures also can increase susceptibility to infection As already mentioned the very use of antibacterial drugs not uncommonly favors the multiplication in vivo of microorganisms which are not susceptible to these drugs probably by first eliminating the normal body flora Extensive surgery and any form of trauma especially if it results in a state of shock is another procedure which can render partially ineffective the defense mechanisms of the body at least for a while

Therefore it is certain that many nonspecific stresses increase the vulnerability of the host, but there is little if any understanding of the mechanisms through which these effects are exerted Indeed many of the relationships that are believed to be so obvious as to need no demonstration in reality have proved to be almost impossible to reproduce in the laboratory For example it is often difficult to increase the susceptibility of animals to infection by nutritional deficiencies temperature changes or emotional upsets More surprisingly experiments in human volunteers fail in many cases to provide clear evidence that exposure to cold and other forms of bad weather increases susceptibility to upper respiratory infections Because of these difficulties it has not yet been feasible to identify the physiologic and biochemical mechanisms through which environmental stresses alter the body response to microbial agents

A few forms of activation of endogenous disease have been consistently reproduced and studied in laboratory models Thus as already mentioned the susceptibility of experimental animals to infections caused by the microbial agents that they normally carry in their tissues can be consistently increased by administration of antibacterial drugs by production of traumatic shock or by extensive body irradiation Treatment with large doses of cortisone is one of the most popular laboratory technics of activation a fact of great importance since administration of this hormone has been a frequent cause of grave clinical accidents in man The infection enhancing effect of cortisone may provide a clue for the analysis of a large and ill understood problem namely the manner in which environmental stimuli including emotional disturbances affect endocrine activity and thereby modify indirectly resistance to disease ACTH also can enhance infection in certain experimental models and its enhancing effect can be neutralized by the proper dose of somatotrophic hormone Injection of thyroid hormone into rabbits markedly increases their resistance to experimental tuberculosis but in contrast hypothyroidism seems to be associated with increased susceptibility to other infections

While it is certain that the course of infectious processes can be altered profoundly by a variety of hormonal influences the mechanisms involved are far from clear The fact that cortisone interferes with the production of antibodies under certain conditions seems at first sight to provide the clue to the problem but this immunologic inhibition cannot account entirely for the infection enhancing effect of the hormone In addition to interfering with antibody production cortisone influences many other physiologic processes some of which may affect directly or indirectly the response of the body to infection Interference with the inflammatory response and with the activity of the reticulo

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of microbial diseases leaves no doubt furthermore that changes can occur in the parasite in the host and in the type of relationship that exists between them That the changes can occur rapidly has been shown with myxomatosis in rabbits and also with lymphocytic choriomeningitis in mice

It is clear in conclusion that the type of relationship existing at any given time between hosts and their parasites is the outcome of many different factors including past racial experience evolutionary adaptation through genetic changes and immunologic processes transient disturbances in the internal and the external environments In the classic infections of exogenous origin the determining etiologic event of the disease is exposure to the infective microorganism In endogenous microbial disease the immediate cause is the *environmental factor* which upsets the biologic equilibrium that normally exists between the host and the microbial agents (persisters) This profound difference in etiologic mechanism suggests that the methods used in the control of microbial diseases both prophylactically and therapeutically must differ from place to place and vary from time to time The methods of sanitation and vaccination that were designed to cope with the great epidemics of the past will not prove to be effective in the control of the disease states caused by microbial agents which are ubiquitous in our communities in the form of dormant infections

So far the main goal of medical microbiology has been to prevent infection from taking place or if it occurs to treat disease once it has become established The technics designed to this end aim at attacking the microbial agents It might be worth considering now whether useful practices of disease control can be derived from the fact that peaceful coexistence with pathogens often occurs in nature This approach will require that the determinants of infection be separated conceptually from the determinants of disease its objective will be to understand and control the processes which are responsible for converting infection into overt disease

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endothelial system disturbance of intermediary metabolism activation of proteolytic enzymes are only a few of the effects of cortisone that might be of importance in this regard

Nutrition is one of the environmental factors for which it is easy to postulate hypothetical mechanisms explaining a role in infectious processes Once pathogens have become established in the body, there come into play a variety of responses that tend to limit their spread beyond the site of the initial lesion One of the essential components of the walling off process is the deposition of fibrin of the sulfated polysaccharides of reticulum and finally of collagen The very nature of this response involving as it does the synthesis of large amounts of special polysaccharides and proteins points to the possibility that it can be interfered with by many metabolic and nutritional disturbances For example deficiency in vitamin C or in dietary sulfur is known to retard the production of reticulum similarly quantitative or qualitative inadequacies in intake of protein or amino acids can handicap the production of fibrin and collagen It has been shown in fact that the rate of healing of wounds is influenced markedly by these dietary factors

There is no doubt that nutritional deficiencies play a decisive role in the prevalence and the severity of microbial diseases among underprivileged people For example the very high susceptibility of patients suffering from kwashiorkor to all sorts of viral bacterial and parasitic diseases is a direct consequence of protein malnutrition However in our own communities it is far more difficult to relate microbial diseases to the nutritional state The problem is further complicated by the fact that paradoxically enough certain nutritional deficiencies seem to increase rather than decrease the resistance to certain viral infections (Reviewed in Dubos and Schaedler 1959, Scrimshaw *et al* 1959)

DETERMINANTS OF MICROBIAL DISEASE

Thus many are the ways in which the total environment can affect the interplay between man and the countless microbial agents which he normally carries or with which he comes into contact This interplay can result in disease or be compatible with the maintenance of health, depending on the environmental circumstances under which the encounter between man and microbe takes place In other words the ecology of microbial disease is under the influence of factors both general and local independent of those which control the frequency of contact with infectious agents To a large extent endogenous microbial diseases are therefore indirectly the expressions of environmental forces

The relative importance of the factors which determine the chance that infection will take place and of those responsible for converting latent infection into overt disease is conditioned naturally by the characteristics of each particular microbial agent but also varies from one population and one community to another When a group of people whatever the race first comes into contact with a pathogen the chance is great that the general mechanisms of resistance will be of little avail and that infection will become manifest in the form of severe disease in a very large percentage of the persons infected This type of situation developed when smallpox measles tuberculosis etc were first introduced among the Amerindians and other primitive people It was produced experimentally by introducing the myxoma virus among the rabbits in Australia and Europe It probably would happen again if yellow fever plague or any type of infections with which Western man has not had wide contact recently were introduced for the purpose of biologic warfare The possibility that mutants of common pathogens can behave as new agents of disease against which the general mechanisms of resistance is of little avail has been suggested to account for the virulence of widespread epidemics like the influenza of 1918 1919

At the other extreme are the epidemiologic situations in which a particular microorganism is ubiquitous in a given community and becomes established in a latent form as a persister in most normal individuals Since the event of infection is not the variable in this type of epidemiologic system the factors that upset the ecologic equilibrium between host and parasite then become the effective determinants of disease Needless to say there are many intermediate situations between these two extremes and the evolution

of microbial diseases leaves no doubt furthermore that changes can occur in the parasite in the host and in the type of relationship that exists between them That the changes can occur rapidly has been shown with myxomatosis in rabbits and also with lymphocytic choriomeningitis in mice

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3

Morphology and Chemical Anatomy of Bacteria*

METHODS

Before 1940 practically all investigations on the morphology and the structure of bacterial cells were performed by ordinary light microscopy using bright or darkfield illumination During the last 20 years several new methods of microscopy have been developed Thus the technics for phase contrast anoptral (Wilska 1954) and fluorescence (Nairn 1962) light microscopy and electron microscopy (Kay 1962) have come into use in bacteriologic research

Under the ordinary microscope living unstained bacteria appear without much contrast against the surrounding medium (Fig 1 A) To enhance the contrast the bacteria may be stained (Fig 1 D) It is also possible to stain selectively certain structures within the bacterial cells (Fig 1 E Fig 2 Fig 28) Before the cells are stained usually they are fixed by heat or chemical agents vital stains are seldom used in bacteriology It is well to remember that fixation and staining procedures may produce artifacts (Murray 1960) Thus structural details appearing in stained preparations should be interpreted with caution

Under the phase contrast and anoptral microscope living bacteria appear with considerable contrast against the background and internal structures may become visible (Fig 1 B) The halo seen around bacteria in the phase contrast microscope is not noticeable when the anoptral microscope is used Dark field illumination especially brightens intracellular inclusions and the outlines of the organisms (Fig 1 C) Structural elements of bacterial cells may also be detected by means of fluorescence microscopy (Fig 1 F)

The resolving power of a light microscope is at best about 0 2 μ when visible light is used for illumination of the object Since most bacteria have a width of only 0 5 to 1 μ the light microscope is not well suited for investigations of structural details of bacterial cells For such studies the electron microscope may be used The resolving power of the electron microscope extends to approximately 1 mμ, i e more than a 100 fold better than that of the light microscope However with the exception of some preliminary experiments (Dupouy Perrier and Durrieu 1960) only fixed specimens have been studied under the electron microscope Thus artifacts may appear in electron micrographs of bacteria as in stained bacterial specimens designed for light microscopy

Electron micrographs of whole untreated bacteria reveal little beyond their size and shape (Fig 8) To obtain information on bacterial subcellular structures the specimens must be sectioned (Figs 15 to 19 24)

* In taxonomic matters *Bergey s Manual of Determinative Bacteriology* ed 7 Baltimore Williams and Wilkins 1957 is followed

Abbreviations used DNA = deoxyribonucleic acid RNA = ribonucleic acid

FIG 1 *Bacillus megaterium* (× 3 000) (A) Ordinary light microscopy (B) Phase contrast (C) Darkfield (D) Cells stained according to Gram (E) Cells stained with Sudan black to show lipid inclusions (F) Cells treated with a fluorescent derivative of polymyxin which becomes bound to the cytoplasmic membrane ultraviolet illumination (Newton B A J Gen Microbiol *12* 226 1955) (G) Cells crushed in India ink (Weibull C Exp Cell Res *9* 139 1955) (H) Cells crushed in water phase contrast (A) (B) and (C) represent the same unstained preparation of living cells (A) (E) and (G) were taken with ordinary illumination

Staining methods especially the negative staining technic has also proved to be useful (Fig 26)

Chemical and physicochemical methods have become increasingly important for investigations on the structure of the bacterial cell The term chemical anatomy is an expression for this trend in bacteriologic research The first step of such an investigation often consists of fragmentation of the cells by mechanical or enzymatic means The homogenate thus obtained is subjected to a differential centrifugation to obtain a certain cell component in a purified state (Figs 7 22) The isolated component is studied subsequently by chemical or physicochemical methods



FIG 2 *Corynebacterium diphtheriae* with stained metachromatic granules (× 3 000)

To localize certain chemical compounds in bacterial cells high resolution radioautography may be used (Fig 30) Bacterial enzymes have also been localized by bombarding parts of the cells with low voltage electrons (Preiss and Pollard 1961)

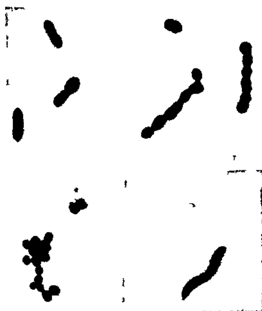


Fig 3 Various types of bacterial cells (× 3 000 phase contrast) (*Top left*) Diplococci (*Top right*) Streptococci (*Bottom left*) Staphylococci (*Bottom right*) A spirillum

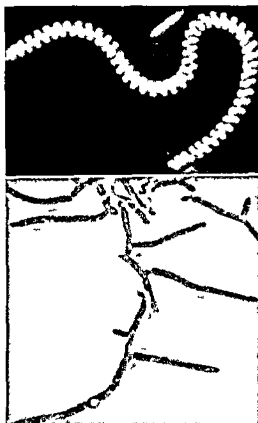


Fig 4 (*Top*) A spirochete (× 3 600 nigrosin staining) (*Bottom*) A branched bacterium (*Nocardia* sp) (× 2 000 phase contrast) (Robinow 1960*a*)

SHAPE SPATIAL ARRANGEMENT AND SIZE OF BACTERIAL CELLS

The discoverer of the bacteria Leeuwenhoek observed that these organisms occur in 3 main forms the spherical the rodlike (cylindrical) and the spiral (helical) Spherical or approximately spherical bacteria (Fig 3 *top* and *bottom left*) are named cocci rodlike ones (Fig 1) bacilli and helical ones spirilla (Fig 3 *bottom right*) or spirochaetes (Fig 4 *top*) Within these main types several subtypes may be discerned Rodlike bacteria are sometimes very short and then may be called coccobacilli Bacilli tapering at both ends are named fusiform Very long bacteria (Fig 5 *left*) are often considered as filamentous rather than rodlike Filamentous bacteria may be branched (Fig 4 *bottom*) Spiral bacteria may form either rigid helices (spirilla) or flexible ones (spirochaetes)

Some bacteria cannot be fitted into any of the main morphologic types mentioned above as for example the stalked bacteria (Houwink 1955 Fig 6) In old bacterial cultures containing a high percentage of dead or devitalized cells irregularly shaped organisms so called involution forms may be seen (Fig 5 *right*)

Most bacteria multiply by binary fission If the 2 cells formed by the fission process separate from each other immediately after they are formed a population of single bacterial cells results Often however the bacterial cells remain attached to one another on one or several subsequent divisions Thus the bacteria may occur in pairs (diplococci or diplobacilli Fig 3 *top left*) or form multicellular aggregates Depending on the mode of division several types of aggregates may result If the multiplication occurs in one plane chains of cells are formed (strepto



FIG 5 A *Mycoplasma* sp (× 2 400 phase contrast) (*Left*) Twelve hour cells (*Right*) One hundred hour cells (Weibull and Lundin 1963)

cocci or streptobacilli Fig 3 *top right*) If cocci multiply in 2 planes at right angles to one another squares of 4 cells may appear (tetrads tetracocci) Cubical packets of cells (sarcinae) may result if the division takes place in 3 planes at right angles to one another Some cocci (staphylococci) divide in several planes at indefinite angles to one another thus forming irregular clusters of cells (Fig 3 *bottom left*) Septa of bacterial cell wall material may not be visible under the light microscope Thus what appears as a single bacillus may consist in fact of several complete cells (Fig 1 F G and H)

Most bacteria especially those of medical interest have dimensions around 1 μ The width (diameter) of bacterial cells ranges from 0 2 or 0 3 μ (the *Mycoplasmatales*) to 3 μ or more (*Caryophanon*) Some spirilla have a length of 20 to 50 μ and filamentous bacteria and free living spirochaetes may be more than 100 μ long

The order of *Eubacteriales* represents the group of bacteria that has been studied most thoroughly from the standpoint of cellular structure However investigations performed on members of other orders e g the *Actinomycetales* (Glauert 1962 Imaeda and Convit 1962) the *Myxobacterales* (Mason and Powelson 1958 Voelz and Dworkin 1962) and the *Mycoplasmatales* (Klieneberger-Nobel 1962) suggest that the main features of cellular anatomy are similar in most sub groups of the class of *Schizomycetes*

FLAGELLA AND FIMBRIAE

MORPHOLOGY AND ARRANGEMENT OF THE FLAGELLA

The flagella form filamentous appendages to the bacterial cells They are between 100 and 200 A thick and usually have a helical shape (Figs 7 to 10) Normally the wave length of the helices is relatively constant within a species or strain Sometimes however a phenomenon called biplicity occurs i e 2 distinct wavelengths are observed one approximately twice the length of the other (Fig 9) Additional variations in flagellar morphology have been described by Leifson (1960 1961)

On account of their thinness individual flagella on living bacteria cannot be observed in the light microscope with ordinary or phase contrast illumination However the flagella have a strong tendency toward aggregation The bundles thus formed may be seen in the phase contrast microscope and still better with darkfield illumination (Fig 10)

Frequently, staining methods have been used for the study of bacterial flagella (Leifson 1960) The diameter of flagella may be grossly enlarged by such procedures On the other hand the staining may not affect the

wave shape of the flagella to any great degree

Two main types of arrangement of the flagella on bacterial cells may be discerned polar and peritrichous flagellation In the former case the flagella originate from one or both ends of the cells (Fig 8 *top right*) in the latter they are distributed at random on the cell surface (Fig 8 *top left*) Generally the flagellated *Pseudomonadales* belong to the former type the flagellated *Eubacteriales* to the latter The various types of flagellation have been discussed in detail by Leifson (1960)

It was thought for some time that the spirochaetes possess flagella However the flagellar structure described by some workers evidently represent detached filaments which are wound tightly around the cell body of living spirochaetes (van Iterson 1956 Weibull 1960 Kawata 1961 Fig 8 *bottom*)

The cytoplasmic origin of flagella is established by the fact that they remain attached to the bacterial protoplast after the wall has been dissolved by lysozyme (Weibull 1960) Several electron micrographs especially of lysed bacteria (Weibull 1960 Fig 8 *top right*) suggest that the flagella are attached to basal granules like flagella of higher organisms



Fig 6 *Caulobacter* sp attached to *Bacillus megaterium* (× 3 500 orig × 6 000) Electron micrograph from Houwink (1955)

Structure and Chemical Composition of Flagella

Chemical investigations on flagella isolated from *Proteus Bacillus Serratia* and *Salmonella* spp (Ambler and Rees 1959 Kobayashi Rinker and Koffler 1959 Weibull 1960) indicate that these organelles consist exclusively of proteinaceous material Only traces of lipids carbohydrates and nucleic acids were found in carefully purified flagella preparations The flagellar proteins which have been named flagellins generally seem to lack the amino acids tryptophan and proline

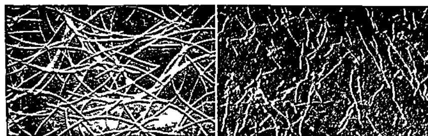


Fig 7 Purified cell components of *Proteus vulgaris* (× 30 000) (*Left*) Flagella (*Right*) Fimbriae Electron microscopy (Weibull and Hedvall 1953)

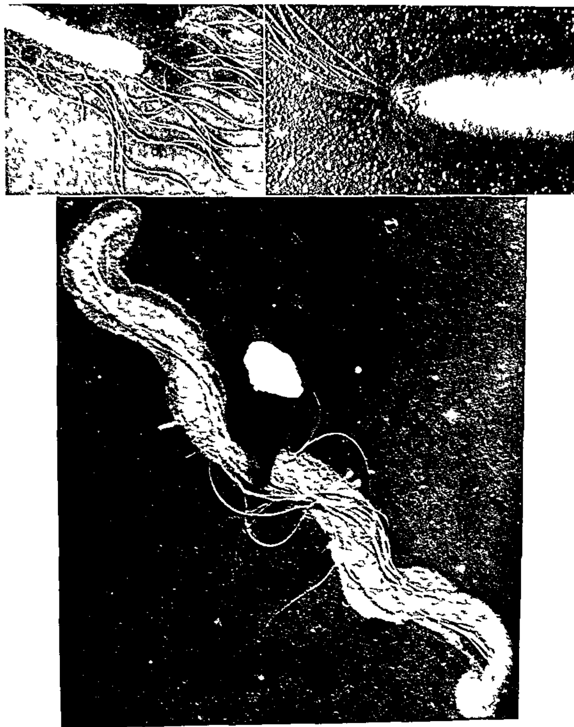


FIG 8 Electron micrographs of unsectioned bacteria (*Top left*) Peritrichously flagellated *Proteus vulgaris* (× 10 000) (Weibull C Biochim Biophys Acta *2* 351 1948) (*Top right*) Polarly flagellated *Spirillum lunatum* with basal granules (× 24 000 orig × 48 000) (Williams M A and Chapman G B J Bact *81* 195 1961) (*Bottom*) A spirochete with filaments wound around the cell (× 22 000) (van Iterson 1956)

X ray investigations have revealed that the bacterial flagella belong to the keratin myosin-epidermin fibrinogen type of fibrous proteins (Beighton Porter and Stocker 1958, Weibull 1960 Burge 1961) Physicochemical studies have shown that the flagella consist of subunits having a molecular weight of approximately 20 000 (Erlander Koffler and Foster 1960) Electron micrographs of sectioned flagella (Kerridge Horne and Glauert 1962) indicate that they do not contain the 9 peripheral and the 2 central subfibrils that are characteristic for flagella of higher organisms rather they appear to consist in some cases of spirally wound subfibrils (Weibull 1960 Marx and Heumann 1962) or of spherical subunits (Kerridge Horne and Glauert 1962)

Function of the Flagella

Most students of bacterial motility have been of the opinion that the flagella are motor organs However some workers advocate the view that the flagella are merely passive appendages of the bacterial cell (Pijper 1957 1962 Winkler and Tscheuschner 1958) The best support to the former view has been given by Stocker and Campbell (1959) who showed that nonlethal treatment which removes the flagella from bacterial cells is associated with loss of motility Motility is resumed when the flagella are resynthesized Hydrodynamic calculations (Weibull 1960 Holwill and Burge 1963) also favor the view that the flagella are motor organs The motility problem has been reviewed in detail by Pijper (1957) and Weibull (1960)

The bacterial H antigens are located in the flagella the proteinaceous nature of the flagella is in keeping with the heat lability of these antigens

Fimbriae

A second type of filamentous appendages of bacterial cells is represented by organelles named fimbriae (Duguid and Wilkinson 1961) or pili (Brinton 1959) They are thinner than flagella and are not curved in regular spirals or waves (Fig 7 *right* Fig 11) Fimbriae have been observed in *Aerobacter Escherichia Klebsiella Proteus Pseudomonas Salmonella* and *Shigella* spp



Fig 9 Cells of *Proteus morganii* stained with flagellar stain Note biplicity (× 1 800) (Leifson 1960)

(Weibull 1960 Duguid and Wilkinson 1961 Marx and Heumann 1962)

Not much is known about the structure and the chemistry of the fimbriae They may contain some proteinaceous material (Brinton 1959) but seem to be resistant to trypsin pepsin and to acid and alkaline environments (Weibull and Hedvall 1953)

The fimbriae do not seem to be related to motility since motile bacteria may be nonfimbriate and nonmotile bacteria may possess fimbriae On the other hand the fimbriae seem to be associated with the ability of bacterial cells to adhere to the surface of other bodies Thus most fimbriate bacteria possess hemagglutinating power whereas nonfimbriate strains are nonhemagglutinating (Brinton 1959 Duguid and Wilkinson 1961)

THE SURFACE LAYERS OF THE BACTERIAL CELL

The peripheral part of the cell may be divided into 3 layers (1) the capsule or

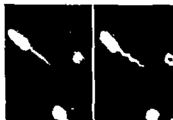


Fig 10 Flagellated cells of *Salmonella typhosa* (*left*) moving (*right*) motionless Sunlight darkfield (× 1 000) (Pijper A J Roy Micr Soc *81* 61 1962)

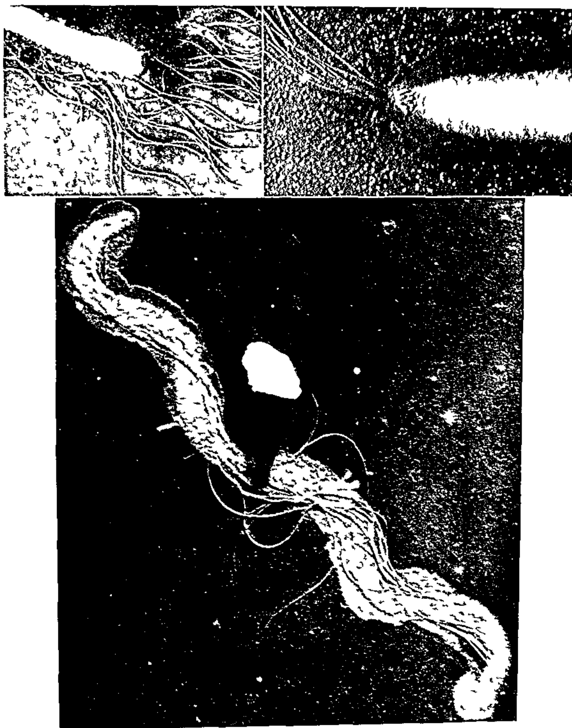


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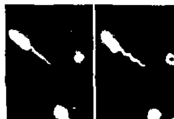


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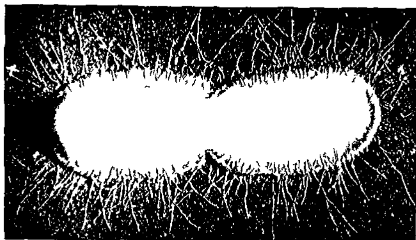


FIG 11 Fimbriate cell of *Shigella flexneri* (× 24 000) Electron micrograph from Duguid and Wilkinson (1961)

slime layer (2) the cell wall and (3) the cytoplasmic membrane The capsule which forms the outermost layer of the cell is difficult to define usually capsules are demonstrable by the light microscope and their removal from a bacterium does not cause the death of the organism The cell wall is commonly defined as the rigid structure which gives a bacterium its characteristic shape The cytoplasmic membrane is considered as a part of the bacterial cytoplasm It is flexible and semipermeable serving to regulate the osmotic pressure inside the cells

Physiologic chemical mechanical or morphologic criteria have been used by various workers to define the surface layers of bacteria Since results sometimes differ depending on the criterion it is difficult to decide with certainty to which of the surface layers certain structures or chemical compounds should be assigned

The streptococcal M proteins for example can be removed enzymatically from living bacteria without killing them (Salton 1953) On this basis the M proteins could be regarded as capsular substances However the removal of these compounds does not change the morphology of streptococcal cell walls (Salton 1953 Slade 1957) Using the terminology of Wilkinson (1958) the M proteins might therefore be considered as microcapsular substances At present no conclusion can be drawn as to the precise location of the M proteins

Many gram negative bacteria lose their O antigens by nonlethal mutations Thus the O antigens might be considered as microcapsular substances since bacteria possessing these substances as a rule do not possess capsules *demonstrable in the light microscope*

Undoubtedly close connections often exist between capsule and cell wall Thus immunologically identical polysaccharides were *found in the wall and the capsule of Bacillus megaterium* strain M (Tomcsik 1956)

With regard to the distinction between cell



FIG 12 India ink wet mounts of *Aerobacter aerogenes* (× 2 000) (*Left*) Cells with small capsules (*center*) Cells with average sized capsules (*right*) Cells with large capsules and loose slime (Duguid 1951)

wall and cytoplasmic membrane high resolution electron micrographs of sectioned non capsulated bacteria show 2 main surface layers (Figs 16 17) Generally the entire outer layer is considered to represent the cell wall the entire inner layer the cytoplasmic membrane Electron micrographs of lysed bacteria (Kellenberger and Ryter 1958 Fig 18) lend support to this view especially as the inner membrane appears as folded and consequently lacking in rigidity

Studies on isolated cell components (Salton 1961) indicate a closer association between cell wall and cytoplasmic membrane in gram negative bacteria than in gram positive ones However plasmolysis experiments (Elo 1937 Robinow 1960a) suggest just the opposite

CAPSULE (SLIME LAYER)

Morphology and Structure of the Capsule

Many procedures for the staining of bacterial capsules have been described These structures can be demonstrated very simply and reliably by suspending living bacteria in India ink (Duguid 1951) The capsules then appear as clear zones around the bacterial bodies (Fig 12) Capsules can also be visualized by adding homologous antiserum to a bacterial suspension producing the so called capsule swelling reaction (Tomcsik 1956)

The size of capsules may vary widely (Fig 12) When the bacteria are surrounded by a diffuse zone of extracellular material (Fig 12 *right*) the terms slime layer or loose slime often are used instead of the word capsule

Bacteria may lose their capsules through mutations Such loss of capsule is reflected in altered appearance of the colonies encapsulated bacteria form more or less mucoid colonies (M colonies) whereas bacteria without capsules form relatively dry colonies with a smooth or rough surface (S and R colonies) In pneumococci encapsulated bacteria usually are designated as S forms bacteria without capsules as R forms The capsules can also be removed enzymatically (Tomcsik 1956)

Most bacterial capsules seem to consist of accumulations of amorphous material However some capsules possess a definite structure Thus capsules containing banded fibrils may be observed in a strain of *Escherichia coli* (Salton 1960a) The capsule of a strain of *Bacillus megaterium* contains a skeletal structure of transverse striations condensed at the poles (Tomcsik 1956 Fig 13)

There exists a close relationship between the presence of capsule in some pathogenic bacteria and their virulence This is especially evident in the case of *Diplococcus pneumoniae Klebsiella pneumoniae* and *Bacillus anthracis* Nonencapsulated strains of these bacteria are avirulent

Chemical Composition of Capsules

Most of the substances forming bacterial capsules are polysaccharides However some capsules consist of material of a polypeptide nature and a capsule containing both polysaccharide and polypeptide is found in *Bacillus megaterium* (Tomcsik 1956) The capsule of *Pasteurella pestis* probably also consists of proteinaceous material associated with carbohydrate (Tomcsik 1956)

Table 1 shows the chemical composition of capsules of some bacteria of medical interest It can be seen that strains of the same species may possess capsules of different chemical composition These differences are reflected in the antigenic properties of the capsules (Heidelberger 1956)

CELL WALL

Methods of Study

Bacterial cell walls have a poor affinity for ordinary stains but according to Tomcsik

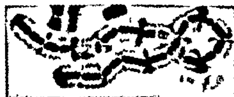


Fig 13 Capsulated cells of *Bacillus megaterium* Note transverse striations in capsule (× 2 500 phase contrast) (Tomcsik 1956)



FIG 11 Fimbriate cell of *Shigella flexneri* (× 24 000) Electron micrograph from Duguid and Wilkinson (1961)

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FIG 12 India ink wet mounts of *Aerobacter aerogenes* (× 2 000) (*Left*) Cells with small capsules (*center*) Cells with average sized capsules (*right*) Cells with large capsules and loose slime (Duguid 1951)



Fig 15 Electron micrograph of *Nocardia calcarea* (thin section) showing "microcapsule" cell wall and cytoplasmic membrane (× 100 000) (Glauert 1962)

the chemistry of isolated cell walls have been studied extensively The wall preparations are obtained by mechanical disintegration of the bacteria and isolation of the wall fragments by differential centrifugation of the homogenate (Salton 1960a Fig 22)

Structure and Chemical Composition of the Walls

In electron micrographs of unsectioned bacterial specimens the cell wall most often appears without any definite structure However regular patterns of approximately spherical subunits have been revealed in the walls of some bacteria (Salton 1960b Glauert 1962 Murray 1962 Fig 14) A fibrous appearance was found in walls of *Bacillus megaterium* (Salton 1960b)

Electron micrographs of sectioned organisms indicate that many or most bacterial cell walls have a multilayered structure (Figs 15 to 19) The walls of gram positive bacteria vary in thickness from 150 to more than 500 A (Glauert 1962) They are thicker than those of gram negative bacteria (thickness 100 A or less) In high resolution micrographs the walls of gram positive organisms appear to consist of 2 thin electron-dense layers separated by a wider layer of slightly less dense material (Fig 16) The walls of gram negative bacteria also appear to consist of 3 layers but in these bacteria all strata have about the same thickness (Figs 17 18) and the density of the middle layer is much lower than that of the surrounding layers

Notable differences exist between gram positive and gram negative bacteria in the chemical nature of their cell walls The composition of the walls of the former group is generally considered to be less complex al



Fig 16 Electron micrograph of *Bacillus subtilis* (thin section) showing cell wall wall septa cytoplasmic membrane mesosome ground cytoplasm and nuclear equivalent (× 95 000) (Ellerling, F A and Romig W R J Ultrastruct Res 6 540 1962)

TABLE 1 CHEMICAL COMPOSITION OF BACTERIAL CAPSULES*

ORGANISM	CLASS OF SUBSTANCE	CONSTITUENTS IDENTIFIED
Diplococcus pneumoniae type II	Polysaccharide	Glucose glucuronic acid rhamnose
Diplococcus pneumoniae type III	"	Glucose glucuronic acid
Diplococcus pneumoniae type XIV		Glucose galactose N acetyl glucosamine
Streptococcus spp groups A and C		Glucuronic acid N acetyl glucosamine
Leuconostoc mesenteroides	Polysaccharide hyaluronic acid	Glucose
Bacillus anthracis	Polypeptide	D glutamic acid
Haemophilus influen ae	Polyribophosphate	Ribose phosphate
Klebsiella spp	Polysaccharide polyuronides	Glucose galactose mannose fucose uronic acid

* Data summarized from Salton (1960a)

and Grace (1955) staining with Alcian blue gives good results However cell walls can be visualized in the light microscope without staining procedures Figure 1 G shows cells crushed in India ink The ink has penetrated into the cells replacing the protoplasm of the living organisms The cell wall thus appears as a light chambered skeleton against a dark background Evidently the walls possess a high degree of rigidity Empty unstained cell walls are also visible in the phase contrast microscope (Fig 1 H) For the study of structural details however the electron microscope must be used (Figs 14 to 19 22)

The rigidity of the cell wall can also be demonstrated by plasmolysis experiments (Elo 1937 Taubeneck 1955 Robinow 1960a Cota Robles 1963) During plasmolysis the outer shape of the cells remains unchanged whereas the protoplasm partly or occasionally completely retracts from the wall

During the last 15 years the structure and

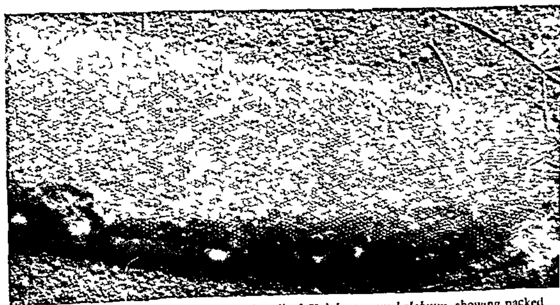


FIG 14 Electron micrograph of whole cell of *Halobacterium halobium* showing packed subunits of cell wall (× 72 500) (Salton 1960*b* from Dr A I Houwink Laboratory of Microbiology Technological University Delft)

though both groups have a so-called basal structure (Salton 1960b) in common This structure could be defined as a mucopeptide or perhaps what is more nearly correct a class of mucopeptides consisting of N acetyl hexosamine N acetyl muramic acid glutamic acid alanine and diaminopimelic acid The last mentioned compound is replaced by lysine in some bacteria e g staphylococci All of the glutamic acid and approximately half of the alanine occurs in the form of the D isomers A tentative model for a subunit of a wall mucopeptide (Salton 1962) is shown in Figure 20 The mucopeptide is generally considered to be responsible for the rigidity of the cell wall (Salton 1960b Martin and Frank 1962 Weidel Frank and Leutgeb 1963 Takeya Hisatsune and Inoue 1963 Fig 22)

Preparative and analytical data indicate that the walls of most gram positive bacteria contain polysaccharides in addition to the basal mucopeptide(s) Thus the group specific carbohydrate of group A streptococci consists primarily of rhamnose and N acetyl glucosamine (Salton 1960a) A polysaccharide consisting of N acetyl glucosamine and glucuronic acid is found in the walls of *Bacillus subtilis* (Perkins 1963) Glucose may be a constituent of the cell wall polysaccharides and is found also in another group of wall substances primarily characteristic for gram positive bacteria the teichoic acids (Baddiley 1962 Baddiley *et al* 1962 Sanderson Strominger and Nathenson 1962) Teichoic acids contain a backbone of ribitol or glycerolphosphate Glucose or N acetyl glucosamine is joined to this backbone by glucosidic linkages (Fig 21) and alanine by ester linkages Teichoic acids do not seem to contribute to the rigidity of the walls but may constitute more than 50 per cent of their dry weight

Only small amounts of lipids and proteins are found in the walls of gram positive bacteria (the streptococcal M proteins form an exception to this rule) The walls of gram negative organisms on the other hand contain proteinaceous material in addition to polysaccharides and the basal mucopeptide Moreover they contain substantial amounts of lipids and perhaps occasionally teichoic acids (Clarke and Lilly 1962)

Complexes of proteins lipids and polysaccharides constituting the bacterial O antigens can be extracted from the walls of gram negative bacteria by diethyleneglycol or some other solvents (Westphal *et al* 1958 Colobert and Creach 1961 Westphal and Luderitz 1963) The antigenic specificity is determined by the polysaccharide moiety or more specifically the terminal oligosaccharides of the polysaccharide chains The smooth (S) forms of *Salmonella* spp contain polysaccharides consisting of glucosamine and galactosamine glucose galactose mannose ribose rhamnose fucose heptoses and dideoxihexoses whereas the polysaccharides of rough (R) forms contain only glucosamine glucose galactose and heptose (Westphal and Luderitz 1963)

The complexes described in the preceding paragraph also represent the endotoxins of

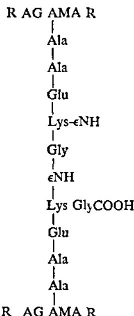


Fig 20 A tentative model of a mucopeptide subunit in *Micrococcus lysodeikticus* cell walls (Salton 1962) AG = acetyl glucosamine AMA = acetyl muramic acid Ala = alanine Glu = glutamic acid Lys = lysine Gly = glycine

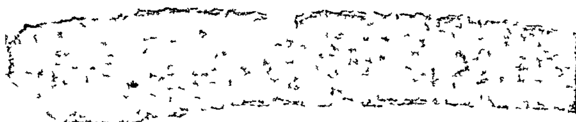


FIG 17 Electron micrograph of *Escherichia coli* (thin section) showing cell wall cytoplasmic membrane ground cytoplasm and nuclear equivalents (× 35 000) (Glauert 1962)



FIG 18 Electron micrograph of lysed *Escherichia coli* cell (thin section) showing cell wall and cytoplasmic membrane (× 100 000) (Kellenberger and Ryter 1958)



FIG 19 Electron micrograph of *Lactobacillus acidophilus* showing thick cell wall cytoplasmic membrane with invaginations and mesosomes (× 60 000) (Glauert 1962)

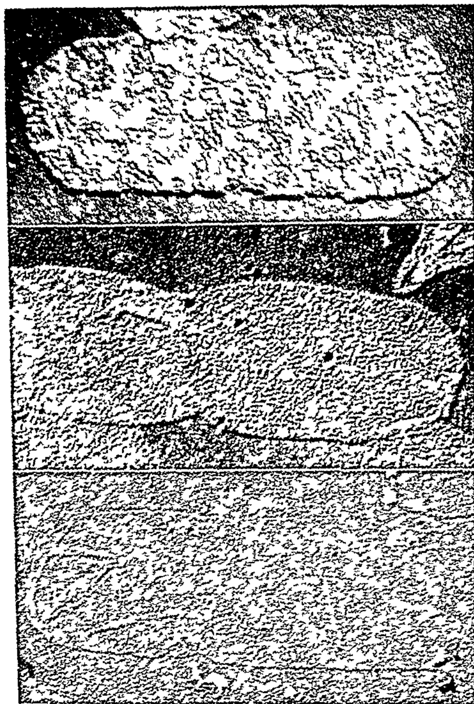


FIG 22 Components of *Escherichia coli* cell walls electron microscopy (× 55 000) (*Top*) Almost undegraded wall (*Middle*) Wall after phenol treatment (*Bottom*) Wall after subsequent pepsin digestion and removal of undigested small fragments The residue represents the rigid mucopeptide-containing layer of the wall (Martin and Frank, 1962)

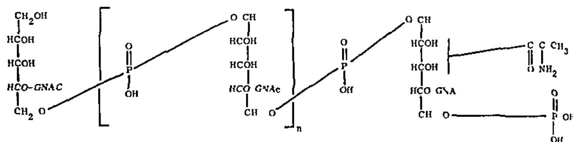


FIG 21 Structure of the teichoic acid of *Staphylococcus aureus* strain Copenhagen The polymer contains 0 5 alanine residue per ribitol the distribution of which is unknown n = 10 to 14 GNAc = acetylglucosamine (Sanderson Strominger and Natheson 1962)

gram negative bacteria It remains uncertain which component of the polysaccharide lipid protein complex accounts for the toxic properties (Westphal *et al* 1958 Westphal and Luderitz 1963 Ribi *et al* 1961)

By combining results from chemical and electron microscopic studies Martin and Frank (1962) concluded that the cell wall of *Escherichia coli* consists of 3 layers an outer lipoprotein layer an intermediate lipo polysaccharide layer and an inner layer con sisting of protein and the rigid mucopeptide (Fig 22) According to Clarke and Lilly (1962) the surface layers of gram negative bacteria apparently consist of 2 unit mem branes of the kind found in higher organisms (Robertson 1959) and a rigid layer in be tween The outer membrane the cell wall probably consists of polysaccharide lipid lipid protein the protein forming the outer most stratum This would be in accordance with the findings of Martin and Frank (1962) It would also be consistent with the appearance of sectioned cells in electron mi crographs (Murray 1962 Figs 17 18)

BIOSYNTHESIS AND FUNCTIONS OF THE CELL WALL

Figure 23 shows a tentative but widely accepted scheme for synthesis of cell wall mucopeptide (Work 1961) Penicillin and some other antibiotics interfere with this syn thesis Usually this process causes the death of the organisms but under controlled condi tions aberrant growth forms the so-called bacterial L forms (Kandler and Kandler 1960 Timakov and Kagan 1961 Kliene-berger Nobel 1962) appear replacing the normal cells Chemical and electron micro scopic studies indicate that many of these bacterial L forms contain no or almost no cell wall constituents (Thorsson and Weibull 1958 Freimer Krause and McCarty 1959 Panos Barkulis and Hayashi, 1959 Kandler and Kandler, 1960 Morrison and Weibull 1962 Tulasne, Minck and Kirn 1962) L form colonies consist of soft elements of a spherical or irregular shape

The biosynthesis and the structure of the cell wall have been reviewed in detail by Salton (1960a and b 1961 1962) Work (1961) Strominger (1962) Rogers (1963) and Perkins (1963)

The cell wall protects the cell very effi ciently from mechanical injury and also pre vents the bacteria from bursting osmotically in hypotonic media The cell wall seems metabolically to be relatively inert As yet no enzyme has been unequivocally localized in it Moreover normal bacterial cells and L forms or protoplasts have roughly the same biochemical capabilities (Kandler and Kandler 1960 McQuillen 1960 Weibull and Beckman 1960 Panos 1962) From one point of view the cell wall can be said to have an unfavorable effect on the survival capacity of the bacterial cell it contains the so-called phage receptors (Salton 1960a) onto which bacterial viruses attach them selves at the beginning of their attack on the host organism The role of the cell wall in sexual conjugation is not clear (Bladen 1963)

BACTERIA NORMALLY LACKING A RIGID CELL WALL

Evidence derived from microscopic and chemical studies indicates that the *Myco*

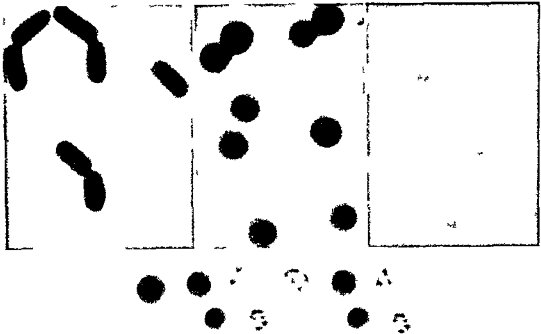


FIG 25 Cells protoplasts and ghosts of *Bacillus megaterium* (× 3 000 phase con trast) (*Top row*) Whole cells protoplasts and ghosts of the M strain (*Bottom*) Proto plasts and ghosts of the KM strain the protoplast on the extreme left lysed between the exposures forming a ghost (The photographs in the top row from Stanier R Y Doudoroff M and Adelberg E A The Microbial World ed 2 Englewood Cliffs Prentice Hall 1963)

plasmatales do not possess a cell wall structure similar to that found in most bacteria (Edwards and Fogh 1960 van Iterson and Ruys 1960 Morrison and Weibull 1962 Fig 24) This is in accordance with the fact that the *Mycoplasmatales* are very easily distorted by mechanical forces (Liebermeister 1960 Klieneberger Nobel 1962 Weibull and Lundin 1963) The *Myxobacterales* seem to possess a cell wall of normal thickness but lacking in rigidity (Voelz and Dworkin 1962)

CYTOPLASMIC MEMBRANE

EXISTENCE OF A CYTOPLASMIC MEMBRANE IN BACTERIA

Plasmolysis experiments carried out mainly on gram negative bacteria offered the first evidence suggesting that bacteria possess a semipermeable membrane surrounding the bacterial protoplasm but located inside the cell wall (Elo 1937 Robinow 1960) Other permeability studies (Stahelin 1954 Weibull 1955 Mitchell and Moyle 1956a and b 1957 Gilby and Few 1959) electron microscopy and staining experiments (Robinow and Murray 1953) also indicated the presence of a semipermeable membrane inside the cell wall of gram positive bacteria These bacteria can be plasmolyzed only with difficulty (Knaysi 1951 Robinow 1960a)

In a few gram positive bacteria the cell wall can be completely dissolved by the enzyme lysozyme If this process is conducted in a medium of sufficiently high osmotic pressure the dissolution of the cell wall does not cause the disintegration of the internal structure of the cell and naked protoplasts are thus released (Weibull 1958 McQuillen 1960 Martin 1963) When the osmotic pressure is lowered the protoplasts disintegrate Conversion of an individual protoplast into a ghost of approximately the same

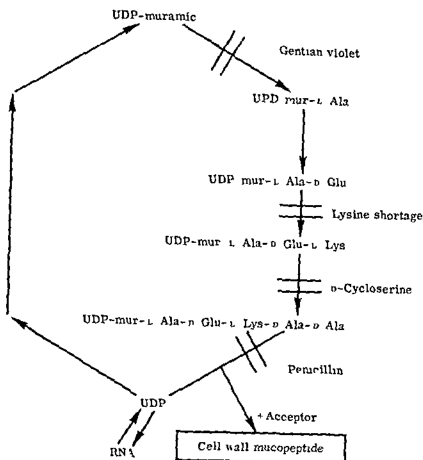


FIG 23 Tentative scheme for the biosynthesis of cell wall mucopeptide in *Staphylococcus aureus* (Work 1961) UDP = uridine diphosphate For other abbreviations see caption for Figure 20



FIG 24 Electron micrograph of *Mycoplasma fermentans* (thin section) showing cell surface layer consisting of single unit membrane (× 180 000) (van Iterson and Ruys 1960)

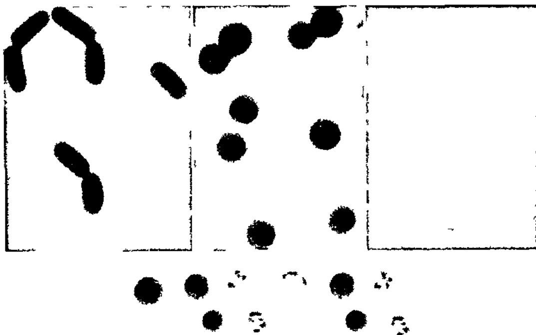


FIG 25 Cells protoplasts and ghosts of *Bacillus megaterium* (× 3 000 phase contrast) (*Top row*) Whole cells protoplasts and ghosts of the M strain (*Bottom*) Protoplasts and ghosts of the KM strain the protoplast on the extreme left lysed between the exposures forming a ghost (The photographs in the top row from Stanier R Y Doudoroff M and Adelberg E A The Microbial World ed 2 Englewood Cliffs Prentice Hall 1963)

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diameter can be followed in the light microscope Essentially, these ghosts represent cytoplasmic membranes containing varying amounts of adherent protoplasmic material (Fig 25)

Modern technics for electron microscopy make it possible to obtain pictures clearly indicating the presence of cytoplasmic membranes in bacterial cells (Figs 15 to 19)

Structure and Chemical Composition of the Plasma Membrane

The cytoplasmic membrane appears in high resolution electron micrographs of sectioned bacteria as a unit membrane (Robertson 1959) consisting of 2 dense strata separated by a less dense layer (Brown Drummond and North 1962 Glauert 1962 Hughes 1962 Murray, 1962 Fig 16) The thickness of the membrane is approximately 75 A Several electron micrographs have been published showing invaginations in the cytoplasmic membrane These invaginations may become deep and wide giving rise to intracytoplasmic bodies still connected with the cytoplasmic membrane (Figs 16 and 19) These structures have been named peripheral bodies chondrioids and mesosomes (Glauert 1962 Murray, 1962 Kawata 1963) and they have been compared with the mitochondria of protozoa and higher organisms (Giesbrecht 1960)

The chemical composition of the cytoplasmic membrane of various bacteria notably *Bacillus megaterium Micrococcus lysodeikticus* and *Streptococcus faecalis* has been studied in some detail Most analyses have been carried out on preparations obtained by lysing bacteria with lysozyme and isolating the membraneous ghosts by differential centrifugation (Gel man Zhukova and Oparin 1959 McQuillen 1960 Salton 1961, Hughes 1962 Shockman *et al* 1963) Some information on membrane composition has also been obtained from studies on mechanically or chemically disrupted bacteria (Mitchell and Moyle 1956a Mathews and Sistrom 1959 Marr 1960a Brown, Drummond and North 1962 Robrish and Marr 1962) It is difficult to assess the extent to which the ghosts or cell fractions represent pure cytoplasmic membranes other cytoplasmic structures, e g the mesosomes mentioned above may be present in the ghosts (Robrish and Marr 1962)

The main constituents of the cytoplasmic membrane seem to be protein (40 to 70% dry weight) and lipid (15 to 40%) Mainly phospholipid containing little, if any, nitrogen has been recovered In some membranes considerable amounts of carbohydrate have also been found (10 to 20%) Substantial amounts of RNA reported in some membrane preparations could represent protoplasmic contamination

Cell wall constituents such as diaminopimelic acid and hexosamine were not found in the membranes of *B megaterium* similarly D amino acids and rhamnose were absent from the membranes of *S faecalis* According to Shockman *et al* (1963) the chemical composition of the membrane of *S faecalis* varied quantitatively with the cultural conditions Freimer (1963) has described recently the chemical composition and the serologic properties of membranes isolated from group A streptococci

Bacterial ghosts' and particulate fractions consisting of the combined surface layers of bacteria exhibit enzymatic activity (Sheinin 1959 Marr 1960a and b Brown 1961 Salton 1961 Abrams and McNamara 1962 Burrous and Wood 1962 Campbell Hogg and Strasdine 1962 Gelman and Lukayanova 1962 Hughes 1962 Ishikawa and Lehninger 1962 Robrish and Marr 1962 Weibull Greenawalt and Low, 1962) Some of these enzymes e g cytochromes and succinic dehydrogenase seem to be associated exclusively with the cytoplasmic membranes or with intracytoplasmic bodies (mesosomes) connected with this membrane (Vanderwinkel and Murray 1962) The localization of enzymes in or near the cytoplasmic membrane has also been demonstrated by topical inactivation of enzymes in whole bacteria (Preiss and Pollard 1961) and by microscopic observations on the sites of tetrazolium and tellurite reduction in normal bacterial cells and L forms (Nermut 1960 Mudd *et al* 1961) On the other hand some enzymes are found mainly in the protoplasmic fraction and others are apparently present in both the membrane and the protoplasm On the whole the cytoplasmic membrane seems to



FIG 26 Electron micrograph of ribosomes from *Escherichia coli* (× 200 000 negative staining) Two types of ribosomes are visible monomers (70 S particles) consisting of 2 unequal subunits and dimers (100 S particles) (Huxley and Zubay 1960)

be rich in enzymes and probably plays an important metabolic role as well as being a regulator of the osmotic pressure inside the bacterial cell These functions of the cytoplasmic membrane have been discussed comprehensively by Mitchell (1961 1962)

CELL DIVISION

The cell division process has been studied with the light microscope (Knaysi 1951 Bisset 1954 Clark Webb and Chance 1957 Robinow 1960a) and with the electron microscope (Chapman 1959 1960 Conti and Gettner 1962 Glauert 1962) In most gram positive bacteria a ring of cell wall material forms and grows inward to form a complete cross wall before division occurs (Fig 16) Perhaps the cytoplasmic membrane (Roth Lewis and Williams 1960) or so called peripheral bodies (Glauert 1962) or other intracytoplasmatic membrane systems (Imaeda and Ogura 1963) play a role in this process In gram positive rod shaped organisms the separation of the daughter cells usually does not follow immediately after the completion of the cross wall consequently chains or aggregates of cells are formed (Figs 1 F G and H)

Cell chains are found less frequently in gram negative bacteria These bacteria usually divide by constriction without the formation of cross walls (Fig 17) However cross wall formation in *Escherichia coli* has been described recently by Conti and Gettner (1962)

CYTOPLASM

The bacterial protoplast can be divided into 3 parts the cytoplasmic membrane the cytoplasm which lacks DNA but is rich in RNA and the nuclear equivalent which contains all or almost all of the DNA of the cell

GROUND CYTOPLASM

In electron micrographs of sectioned bacteria (Figs 16 and 17) the cytoplasm usually appears to be homogeneous and finely granular The size and the shape of the cytoplasmic granules as a rule termed ribosomes are difficult to ascertain from inspection of sectioned material but in most cases they appear to be roughly spherical with a diameter of 100 to 300 Å (Murray 1960 Glauert 1962 Brieger 1963) The appearance of the sectioned cytoplasm seems to vary depending on the cultural conditions under which the bacteria are grown (Costerton Murray and Robinow 1961 Vanderwinkel and Murray 1962)

Detailed information concerning the nature of bacterial ribosomes has been obtained from electron microscopic chemical and physicochemical studies on material isolated from disintegrated cells Thus in extracts of *Escherichia coli* Tissieres *et al* (1959) Hall and Slayter (1959) and Huxley and Zubay (1960) found particles having sedimentation coefficients of 30 50 70 and 100 S The particles contain 80 to 90 per cent of the RNA of the cells The 100 S particles represent dimers of the 70 S particles and each 70 S particle consists of one 50 S and one

30 S component (Fig 26) The 50 S particles are approximately spherical having a diameter of approximately 150 A and a molecular weight of 1,800 000 The recovery of the various particle aggregates depends on the concentration of Mg^{++} ions in the suspending medium According to Bowen *et al* (1961), mainly 50 S and 30 S particles are present in living cells of *E coli* and *Bacillus cereus*

The main components of the *E coli* ribosomes are RNA (Kurland 1960) and protein (Waller and Harris 1961) According to Zubay and Wilkins (1960) the nucleic acid and the protein of the ribosomes have an appreciable degree of structural independence as in nucleohistones

Ten to 20 per cent of the bacterial RNA the so-called soluble RNA has a relatively low molecular weight approximately 25,000 and is not bound to protein (Tissières 1959) This RNA is also termed transfer RNA since it transports amino acids to the sites of protein synthesis the ribosomes in the bacterial cells (Hunter and Godson 1961 Perutz 1963 Zubay 1963) Another kind of RNA messenger RNA carries genetic information from DNA to the ribosomes Messenger RNA is metabolically unstable and has a molecular weight of 250 000 or more (Risebrough Tissières and Watson 1962) It constitutes at the most a few per cent of the total RNA of the cell which in turn represents 10 to 25 per cent of the bacterial dry weight depending on the physiologic age of the organisms and the cultural conditions

The major part of the cytoplasmic proteins which are not associated with nucleic acids or located in the cytoplasmic membrane have sedimentation coefficients between 3 and 6 S (Schachman Pardee and Stanier 1952 Koenig *et al* 1959) Most of the enzymic activities of the ground cytoplasm seem to be located in these proteins However nucleases have been found in the ribosomes of *E coli* and *Azotobacter vinelandii* and in the ribosomes of the former organism a peptidase has also been demonstrated (Marr, 1960b)

A number of low molecular weight substances such as coenzymes vitamins nucleotides, peptides amino acids and sugars are also present in the ground cytoplasm

Bacterial cytoplasm, which contains approximately 80 per cent water seems to represent a sol rather than a gel Thus the ultracentrifugation experiments of King and Beams (1942) showed that the cytoplasmic inclusions of *Spirillum volutans* were displaced to the dependent parts of the cells upon centrifugation at 400 000 G When the cells were removed from the centrifugal field, the inclusions redistributed within 30 to 60 minutes It has also been noted that the lipid granules of *Bacillus megaterium* cells change their intracellular position rapidly (Robinow 1960a)

There are indications that the salts and other low molecular weight compounds of the cytoplasm exist to a large extent in a free solution instead of being bound to the cytoplasmic macromolecules Thus Mitchell and Moyle (1956a) found a good agreement between the values for the intracellular osmotic pressures of *Staphylococcus aureus* and *E coli* obtained from direct measurements and from estimations of the contents in the cell of low molecular weight compounds Similarly Sistrom (1958) reported that galactosides enzymatically transported into *E coli* cells exhibited an intracellular osmotic pressure that could be explained only if the accumulated galactosides existed in free solution

Cytoplasmic Inclusions

Bacterial cytoplasm does not contain organelles comparable with the endoplasmic reticulum Golgi apparatus or lysosomes seen in mammalian cells

In bacteria of medical interest the following kinds of inclusions are found lipid inclusions (Burdon 1946) granules of glycogen and starch (Murray 1960) and metachromatic granules In some bacteria sulfur granules and protein crystals occur (Murray 1960 Robinow 1960a) The presence in bacteria of vacuoles containing gas or liquids has also been demonstrated (Houwink 1956 Murray 1960 Brieger 1963)

Metachromatic granules (Fig 2) owe their name to the fact that certain dyes e g toluidine blue and methylene blue change color when combining with these granules Metachromatic inclusions are also named

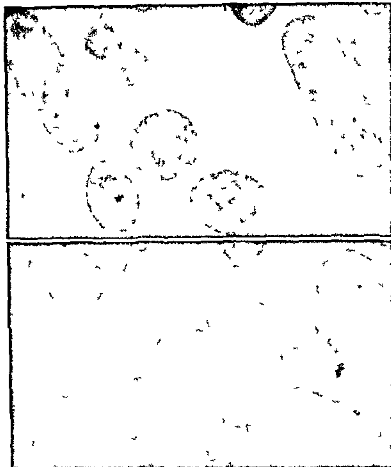


FIG 27 Electron micrographs of *Escherichia coli* cells (thin sections) (*Top*) Cells containing 17 4 per cent and (*bottom*) 4 5 per cent glycogen (× 30 000) (Holme and Cedergren 1961)

volutin because they occur prominently in cells of *Spirillum volutans* Histochemical observations and chemical analyses on material extracted from bacteria indicate that the main constituent of the metachromatic granules is polymetaphosphate (Winkler 1956 Sall Mudd and Takagi 1958 Murray 1960) The metachromatic granules have been identified with nuclear equivalents and intracellular reduction sites However these identifications are evidently not tenable (Glauert and Brieger 1955 Winkler 1956 Mudd Takeya and Henderson 1956)

Lipid granules seem to be the most common type of cytoplasmic inclusion of lipid nature Chemical analyses indicate that these granules contain poly β hydroxybutyric acid (Hayward Forsyth and Roberts 1959 Doudoroff and Stanier 1959 Wilkinson 1959 Murray 1960 Slepecky and Law 1961 Fig 1 B C and E) and some other lipid in addition Their sudanophilic character probably should be attributed to the latter component (Wilkinson, 1959)

The accumulation of glycogen granules in *E coli* cells has been studied in some detail by Holme and Cedergren (1961) A great number of low density areas (holes) are seen in electron micrographs of cells rich in glycogen whereas in cells containing little glycogen only few holes are seen (Fig 27) Cells rich in glycogen show heavy staining with the periodic Schiff reagent

Cells of *Clostridium* spp accumulate a starchlike substance named granulose at the time of sporulation (Murray 1960 Robinow 1960a)

The cultural conditions and the physiologic age of the organisms greatly influence the occurrence of cytoplasmic inclusions in



Fig 28 *Bacillus cereus* stained according to Feulgen Note dark nuclear equivalents (× 3 500) (Robinow 1960a)

bacteria It has been suggested that these inclusions represent waste products or storage material In the case of poly β hydroxybutyric acid evidence favoring the latter view was presented by Wilkinson (1959 1963) and Doudoroff and Stanier (1959) The findings of Holme and Palmstierna (1956) indicate that glycogen represents storage material in *Escherichia coli* Sall Mudd and Takagi (1958) found that polymetaphosphate was used up during periods of cell division in synchronized cultures of *Corynebacterium diphtheriae*

NUCLEAR EQUIVALENT

Existence and Methods of Study

Clear evidence for the existence of nuclear bodies in bacteria was not presented until comparatively recently mainly because the importance of selecting appropriate bacterial specimens for the experimental work was not well understood and because most methods for observing or staining nuclei in higher organisms are unsuitable for bacteria However in the late 1930s positive results were obtained by Feulgen staining a reaction considered to be specific for DNA (Fig 28) In later studies use was made of other dyes which stain nuclear material intensely For example the nuclear bodies fluoresce specifically when stained with acridine orange (Robinow 1956 1962 Hayes 1960 Kellenberger 1960 Brieger 1963)

Usually the nuclear equivalents of living bacteria are seen poorly or not at all in the light microscope However when the organisms are suspended in a medium of a high refractive index often the nuclear bodies can be observed in the phase contrast microscope and their division can be followed (Fig 29)

Early electron micrographs did not give very informative pictures of the nuclear equivalents In electron micrographs of bacteria sectioned fixed and embedded according to improved methods a central region filled with delicate fibers can be seen (Figs 16 and 17) Comparisons with autoradiographic and light microscopic material show that this region contains the nuclear material (Birch Andersen 1955 Caro and Tubergen 1962 Fig 30)

Chemical analyses on whole bacteria and isolated nuclear bodies indicate that all or almost all of the bacterial DNA is located in the nuclear region (Hayes 1960)



Fig 29 Division of cells and nuclear equivalents in *Escherichia coli* (× 2 000 phase contrast) Growth medium contained 20 per cent gelatin Time between exposures 3 to 9 minutes (Dr D J Mason Department of Microbiology The Upjohn Company Kalamazoo Mich)

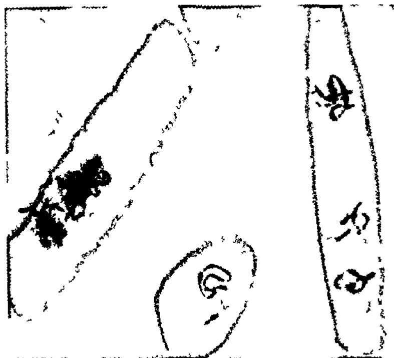


FIG 30 Thin sections of *Bacillus subtilis* labeled with (H^3) thymidine The photographic grains seem closely associated with the nuclear regions (× 38 000) (Caro and Tubergen 1962)

MORPHOLOGY STRUCTURE AND CHEMICAL COMPOSITION

The light microscopic appearance of the nuclear regions of bacteria is extremely variable Thus they may appear as round bodies dumbbells axial filaments or scattered granules connected with fine strands This variation is often related to cultural conditions or the fixation methods used but it may also be ascribed to generic or specific differences In any case all workers agree that chromosomes of the morphology characteristic for higher organisms do not occur in bacteria nor is there a mitotic apparatus (Hayes 1960 DeLamater 1962)

The fine structure of nuclear bodies in bacteria has been well studied with the electron microscope No membrane is seen between the nuclear bodies and the cytoplasm (Figs 16 and 17) This is the main reason along with the absence of mitosis for the frequent use of the term nuclear equivalent instead of nucleus when referring to bacteria. Nuclear material in bacteria commonly consists of fibrils 20 to 60 A thick which fill the entire nuclear region (Figs 16 and 17) The pictures published by Giesbrecht (1962) suggest that in some bacteria at least the fibrils are organized in coils similar to those found in the chromosomes of some protozoa Close associations between the nuclear bodies and mesosomes have also been described (Giesbrecht 1962 Robinow 1962)

A wealth of genetic data (see chapter on bacterial genetics) indicates that the genes of *Escherichia coli* are located on a single chromosome which usually forms a closed loop A morphologic confirmation of this genetic model has been presented by Cairns (1963) who prepared autoradiographs of isolated nuclear material previously labeled with (H^3) thymidine (Fig 31) The length of the chromosome is approximately 1 000 μ which is in accordance with genetic data A chromosome of this length must necessarily be coiled or bent repeatedly when located inside the cell Hypothetical models explaining the structure of the nuclear bodies have been presented by Kellenberger (1960) Schlote (1961) and Robinow (1962)

The findings of Kleinschmidt *et al* (1961) Cairns (1963) and others indicate that the bacterial DNA like the DNA of higher organisms exists in the form of a fiber which on the molecular level has the double helix structure presented by Watson and Crick



Fig 28 *Bacillus cereus* stained according to Feulgen Note dark nuclear equivalents (× 3 500) (Robinow 1960a)

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these organelles within the cytoplasm usually one spore is formed in each cell Several morphologic spore types exist which may be used for taxonomic purposes Thus there exist spherical ellipsoidal and cylindrical spores which may be located terminally subterminally or centrally in the mother cell (the sporangium) Spores may have a diameter greater than that of the vegetative cells causing the spore forming part of the organisms to bulge (Fig 32) The spores have a high refractive index and are thus easily recognized under the light microscope They stain only after heating but once stained tend to retain the dye to a far greater extent than vegetative cells

The structure of the endospore varies somewhat from one species to another but generally the central part of the spore the spore core appears without much structural detail in electron micrographs (Fig 33 C) The core is surrounded by a double membrane the spore wall or spore cytoplasmic membrane (SM) Outside this membrane is a layer named the cortex (CX) which in turn is surrounded by 1 or 2 spore coats (IC and OC) The inner spore coat often is laminated The spore coats may be covered by a so-called exosporium The exosporium and the outer spore coat often are separated from each other either completely or at the poles of the spore (Robinow 1960b Tomcsik 1962)

The chemical composition of bacterial spores differs in many respects qualitatively or quantitatively from that of the corresponding vegetative cells The most striking difference is represented by the high content (approximately 10%) of dipicolinic acid (pyridine 2 6 dicarboxylic acid) in spores Vegetative cells are devoid of this compound (Halvorson 1962) The water content of spores as compared with that of vegetative cells has been investigated repeatedly but there is some discrepancy in the results obtained Measurements of the refractive index of spores indicate that they contain only 10 to 20 per cent of water whereas according to direct estimation of their moisture content and permeability measurements the difference in water content between spores and vegetative cells is not very marked (Black and Gerhardt 1962 Halvorson 1962)

Mucopeptide material similar to that found in walls of vegetative cells is also found in spores It is probably located in the cortex The spore coats seem to contain chiefly proteinaceous material (Warth Ohye and Murrell 1963)

Many enzymes have been found in bacterial spores (Halvorson 1962)

Marked changes occur in the enzyme pattern during formation of spores (sporulation) Thus cytochromes are not found in spores of *Bacillus* spp whereas vegetative cells are rich in these enzymes

Serologic investigations (Halvorson 1962 Tomcsik 1962) show that antisera prepared against surface structures of *Bacillus* spores do not react with the corresponding vegetative cells

The sporulation process has been characterized in considerable detail from the morphologic point of view (Fitz James 1960 Robinow 1960b Ohye and Murrell 1962 Young and Fitz James 1962) The first rec

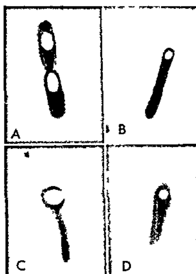


FIG 32 Spore forming bacteria (× 3 000 phase contrast) (A) *Bacillus megaterium* with central and terminal ellipsoidal spores (B) and (C) *Clostridium* spp with terminal spherical spores (D) An unidentified bacterium with a terminal spherical spore

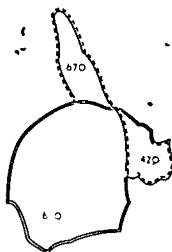


Fig 31 Autoradiograph of dividing *Escherichia coli* chromosome labeled with (H^3) thymidine for 2 generations Length of chromosome approximately 1 100μ (length of scale 100μ) In part of the chromosome only one DNA strand is labeled in part both strands (see explanatory drawing) (Cairns J Sympos Cold Spring Harbor *28* 43-46 1963)

(1953) Chemical components other than DNA have not yet been definitely localized in the nuclear equivalents of bacteria Analyses carried out on isolated nuclear bodies of *Bacillus megaterium* (Spiegelman Aronson and Fitz James 1958 Ezekiel 1961) indicate that they may also contain RNA and protein According to Spiegelman Aronson and Fitz James the nuclear protein represents a core around which the DNA is layered On the other hand Wilkins and Zubay (1959) concluded that the DNA of *E coli* unlike the DNA of higher organisms probably is not bound to protein

BACTERIAL RESTING FORMS

Endospores

The formation of endospores is especially characteristic for the genera *Bacillus* and *Clostridium* The vegetative cells develop

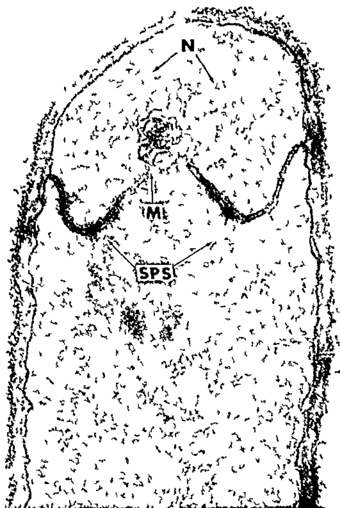


FIG 35 Electron micrograph of *Bacillus coagulans* (thin section) showing a stage in the sporulation process (× 170 000) SPS = spore septum M = mesosome N = nuclear material (Ohye and Murrell 1962)

ognizable step consists of an invagination of the sporangial cytoplasmic membrane (Fig 34 A) In this way a pocket is formed which contains the nuclear material of the future spore and some cytoplasm (Fig 34 B) The intrusions of the cytoplasmic membrane come into contact with the nuclear material and a mesosome appears in the same region (Fig 34 B Fig 35) When the cytoplasmic septum or spore septum has completely traversed the cell (Fig 34 D) it begins to bulge (Fig 34 E) and 2 or 3 mesosomes appear outside the developing spore (Fig 34 F to H) The later stages of the sporulation process are charactertized by deposition of cortical material between the 2 layers of the spore septum The high refractility of the spore develops at this stage The inner layer of the spore septum eventually forms the spore plasma membrane (spore wall) The outer layer develops into the spore coats and the exosporium

The first step in the germination process which gives rise to a new vegetative cell is a loss of refractility and swelling of the spore The young vegetative cell then extrudes through the bursting spore coats The wall of this cell seems to be the former spore wall Electron micrographs show that in the core of the germinating spore regions appear containing nuclear material of the kind found in vegetative cells Simultaneously the cytoplasm becomes distinctly granular (Robinow 1960b)

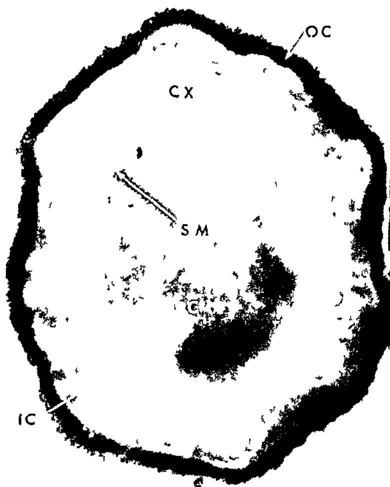


FIG 33 Electron micrograph of a mature spore of *Bacillus coagulans* (thin section) Spore heated for 8 minutes at 110 C (× 90 000) The micrograph shows the spore core (C) spore cytoplasmic membrane or spore wall (SM) cortex (CX) spore cytoplasmic membrane or spore wall (SM) cortex (CX) inner spore coat (IC) and outer spore coat (OC) (Dr D F Ohye CSIRO Division of Food Preservation Ryde New South Wales Australia)

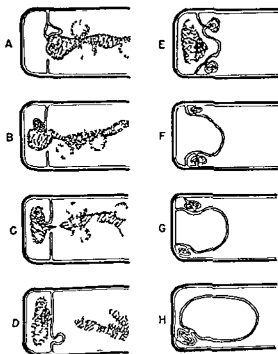


FIG 34 A diagrammatic representation of the early stages in the sporulation process in bacilli (Fitz James *1960) For explanations see text*

teria visible in the ordinary light microscope the preparation is treated with a counterstain e g neutral red safranin or Bismarck brown

Many theories have been advanced to explain the Gram stain reaction It has often been proposed that the dye iodine complex is bound to some specific compound in the bacterial cells A number of substances have been discussed in this connection especially nucleic acids lipids and proteins Some workers have assumed that gram positive organisms have a lower isoelectric point than the gram negative ones and consequently a stronger affinity to the primary basic Gram stain

However it has become increasingly clear that the Gram stain reaction cannot be explained in simple chemical or physicochemical terms The structural architecture of the bacterial cell must also be taken into account The most recent investigations (Salton 1963 Scherrer 1963a and b) point to the permeability of the cell wall as being of decisive importance Thus the wall of gram positive organisms becomes largely impermeable to low molecular weight compounds when the organisms are suspended in 70 to 100 per cent alcohol Thus probably the alcohol soluble dye iodine complex is trapped within the wall in gram positive bacteria with the consequence that the cells remain stained In gram negative organisms on the other hand the complex rapidly leaks out through the wall

It should be emphasized that there is no strict partition line between gram positive and gram negative bacteria the term gram variable is often encountered Many gram positive organisms tend to become gram negative with increasing physiologic age

The Acid Fast Stain

Certain bacteria especially *Mycobacterium* spp are not penetrated readily by ordinary dyes However when they are treated with a solution of a phenylmethane dye (usually basic fuchsin) in aqueous phenol preferably in combination with heating staining is achieved Once stained the mycobacteria retain the dye even when treated with dilute mineral acid hence the term acid fast

It has been assumed (Lamanna and Mallette 1959) that phenylmethane dyes in combination with phenol are more soluble in the milieu provided by the cell constituents of mycobacteria than in the decolorizers used This solubility might be due to certain lipids present in mycobacteria Mycobacteria with crushed walls lose their acid fastness probably the intact wall prevents the extraction of dye from the cells by the decolorizer

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Cysts and Conidia

The cysts do not develop within the cyto plasm but arise by a rounding up of the en tire mother cell The cysts of *Azotobacter* spp are covered by a multi layered envelope outside the ordinary cell wall (Tchan Birch Andersen and Jensen 1962) Cysts or cyst like forms have also been described in spiro chaetes (DeLamater *et al* 1951) and spi rilla (Williams and Rittenberg 1956)

The formation of conidia or conidiospores i e resting forms produced at the end of filamentous cells is especially characteristic for the family of *Streptomycetaceae* With re gard to formation and the structure the conidia resemble bacterial cysts rather than endospores (Glauert 1962)

Intracellular Granules and Filterable Forms

Many workers have described small gran ular elements found in bacterial cultures either free or inside other bacterial forms (Klieneberger Nobel 1951 1962 Bisset 1955) These granules often pass filters which normally retain bacteria It has been assumed that they have a reproductive func tion but this view has been disputed (Kellen berger Liebermeister and Bonifas 1956 Weibull 1963)

BACTERIAL STAINS

General

Most of the staining methods mentioned in the preceding sections specifically stain certain subcellular components of the bac teria e g the nuclear equivalents These methods are used only to a limited extent in diagnostic work However other staining procedures such as the Gram staining tech nic are of great value for diagnostic pur poses When these methods are applied either the entire bacterial cell (or rather the protoplast) is stained or it is not stained at all depending on the taxonomic position of the species studied Certain simple com pounds, e g methylene blue and basic fuch sin stain almost all kinds of bacteria and are used to detect bacteria in general

Simple Stains

The main protoplasmic constituents of all bacteria are negatively charged except at rather low pH values Thus stains owing their specific light absorption to colored positive ions are well suited for staining most bac terial cells The practical performance of these staining procedures and of more com plicated staining technics is described in bacteriologic manuals (Conn 1957 Cruick shank 1960)

The Gram Stain

The Gram staining procedure (Bartolo mew and Mittwer 1952 Scherrer, 1963a) consists of 5 steps

1 The cells are fixed Heat fixation is the standard method but the method of fixation is not of decisive importance for the result of the staining

2 The cells are stained with the so-called primary Gram stain The standard dye used is crystal violet (hexamethyl triamino tri phenylmethane hydrochloride) Homologues of this dye (methyl violets) and some other dyes can also be used but no compound has been found which is superior to crystal violet The primary stain is fixed to both gram positive and gram negative bacteria The dye seems to be distributed uniformly in the cyto plasm (Tchan 1963)

3 The bacteria are mordanted with an *iodine potassium iodide solution This causes the formation of a complex which is insolu* ble in water of primary stain and iodine The *mordanting cannot be carried out before the* application of the primary stain

4 A decolorizing agent is applied to the stained and mordanted preparation This causes an extraction of the dye iodine com plex from the group of bacteria which is called gram negative whereas in the gram positive bacteria the complex is retained

Usually 70 to 100 per cent alcohol is used as decolorizer Prolonged treatment with de colorizer also extracts the dye iodine com plex from gram positive organisms Thus the difference between gram positive and gram negative bacteria is of a quantitative rather than qualitative nature

5 In order to render gram negative bac

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4

Bacterial Genetics

Bacteria generally increase their number by the division of the cell body into two Following a period of growth both daughter cells can divide further By subsequent growth and divisions this can give rise to an enormous number of organisms in a brief period of time Barring an occasional genetic accident i e a mutation all of the descendants of a single bacterium will be identical with respect to all of the characteristics of the original ancestor The material components of the individual organisms within a bacterial culture are associated only up to the moment of cell division Therefore the members of the bacterial community are in *complete isolation one from the other with* no avenue of communication

Up until a little over a decade ago the above description would have been sufficient to account for the events of bacterial multiplication Since that time it has been found that for some bacteria there are channels of communication between the organism through which may pass information of the most useful sort—genetic information Genes (those factors which specify the properties *of an organism*) can sometimes travel either in large or small blocs from bacterium to bacterium The advantage of gene transfer is that two lines may *on contact reassemble* their features in such a way as to synthesize an organism more highly adapted to a particular environment In higher forms gene exchange occurs by sexual reproduction In bacteria, as we shall see, a number of different mechanisms for gene transfer have been found These different kinds of mechanisms for gene transfer in bacteria may represent evolutionary experiments for the *development of gene transfer systems*

The sciences of bacteriology and genetics were born some 100 years ago They proceeded to develop quite independently of each other until a marriage that was most fruitful for both sciences was made in the middle forties In a sense it was a marriage of desperation each science needed the other The problem of bacterial variation plagued a rational approach to the control of bacteria and the understanding of fundamental bac*terial growth processes In genetics the* formal gene had been described located on the chromosome and carefully mapped The problems of the chemical nature of genetic material and how it functioned were unapproachable with the then current technics

This is not to imply that there were no attempts at a genetics of bacteria for microbiologists pondered on the mechanism of variation but since the organisms seemed to reproduce solely by vegetative growth both the microbiologists and the geneticists saw no rational approach to the problem

Consideration of any biologic organism *and its hereditary mechanism leads to two* different points of view One, analogous to the genetic system that has been described for *higher forms states that an organism* has a specific well isolated unit of genetic material which contains the essential in

formation for the cell syntheses and growth The second point of view which held sway for some time in microbiology despite the lack of precedent is that each subcellular unit is responsible not only for its own function but also for its own reproduction

The latter hypothesis is necessarily vague as it is never clear to what level of cellular organization it refers—organelle substructure molecule or atom Also arising from the second point of view is the idea that variation is due to the direct action of the environment on the unit which is responsible for the particular trait in question Resistance to a drug is due to the direct action of the drug on some subcellular unit Thus the terms *adaptation* and *training* crept into the bacteriologic literature

Although the experiment which would have disposed of the second hypothesis was initiated as early as 1928 (bacterial transformation) it was not analyzed completely until 1944 and its significance was not grasped until almost 1950

BACTERIAL VARIATION

The experimental approach to the analysis of bacterial variation was hindered by the failure to differentiate carefully between the variation of cultures as a whole and the variation of the individual organisms that made up the culture The appearance of an enzyme such as betagalactosidase in an *E coli* culture following the addition of the substrate lactose and the production by certain non lactose fermenting cultures of a few individuals capable of fermenting lactose are two quite different phenomena It is a culture of individuals of the latter class that exhibit the former phenomenon The remarkable success that has come to bacterial genetic analyses has in the main derived from careful separation of these two elements

The components necessary for a bacterial genetic analysis are cultures of individuals which are obtained by simple procedures from single organisms of the parental culture Because of the enormous numbers of individuals that can be grown in a short time and the general availability of procedures with a high degree of selectivity it is often possible to find unique and rare individuals For instance it is possible in 10^{10} organisms to find the one that is resistant to the drug streptomycin by plating large numbers of bacteria in the presence of the drug

With this conceptual approach Luria and Delbruck analyzed the mutation of *E coli* from phage sensitivity to phage resistance If the mutation was an adaptive response to phage infection then the sampling of one culture should give the same number of mutants as would the sampling of many independent cultures of the same kind On the other hand if the mutations occurred independently of the selective agent (the bacteriophage) as a random discrete process with a constant probability per bacterial generation then mutation would occur at different times during the growth of different cultures and give rise to a variable number of mutant descendants prior to the sampling The results of Luria and Delbruck indicated that the mutants occurred prior to and independent of the selective agent and that the only role of the selective agent was precisely what the term implies i e it selected the mutant forms

Another useful procedure leading to the same conclusion employed replica plating and an indirect selection of mutants (Lederberg and Lederberg) Bacteria were placed on a nutrient agar medium and allowed to grow to confluence If a mutation occurs during the growth of this culture the descendants of this mutant will be located at a fixed site on the agar surface By application of a piece of velveteen fabric about one third of the growth is removed and this is used to inoculate several other dishes containing a selective medium From the position of the mutants on the replica plates the position of the mutants on the master plate which never have been exposed to the selective agent can be inferred This area can be picked and replated and the process repeated Since only a small area of the master plate is picked relative to the total area of the plate this results in a considerable concentration of the mutant By repeating the procedure and plating smaller and smaller amounts of the area picked the size of the mutant clone grows larger and larger before exhaustion of the medium stops growth Ultimately a pure

clone of mutant organisms can be obtained in which neither these microorganisms nor any of their direct ancestors have been exposed to the selective agent

A procedure also is available for recovering mutants that have lost their capacity to synthesize particular substances The antibiotic penicillin only kills growing bacteria When a culture which contains mutants that require a particular amino acid for growth is inoculated into a medium which lacks this amino acid and contains penicillin the non mutant cells grow and are killed

Other ingenious procedures have been developed to study the nature of mutation and all have led to the same conclusion mutation is a spontaneous occurrence and independent of the nature of the selective agent This is not to say that mutation rates are independent of the organism s environment Quite a number of reagents will increase the mutation rate but they do so in a more or less nonspecific manner in that they affect many different genes

GENETIC TRANSFORMATION

The essential requirements for study of genetic recombination in any type of bacteria are (1) two potential parental lines of the organism which differ by at least one well-defined and readily recognizable property and (2) a means for selecting against at least one of these lines

The first experiment involving gene transfer in bacteria was probably that reported by Griffith in 1928 Mice are so susceptible to certain encapsulated (smooth or S) pneumococci that intraperitoneal injection of a few organisms will cause a fulminating infection which kills the mouse In contrast nonencapsulated (rough or R) pneumococci or heat inactivated pneumococci whether rough or smooth do not cause disease when injected into mice Griffith injected intraperitoneally a mixture of heat killed S pneumococci and viable R pneumococci The R pneumococci were derived from a different capsular type than the S The mice infected with this mixture died and living encapsulated pneumococci of the heat killed S type were recovered from them Both of the elements for a gene transfer experiment were present the differentially marked strains and the selective agent (in this instance the mouse) Although it was not recognized at this time gene transfer had occurred The resolution of the nature of this transforming system came in two stages In 1944, Avery McCarty and MacLeod showed that the material that caused the transformation of unencapsulated forms to encapsulated forms was deoxyribonucleic acid (DNA) The strictly genetic interpretation of this result awaited the demonstration that other pneumococcal properies such as drug resistance and M proteins (Hotchkiss) could be similarly transferred Physiologic functions as diverse as resistance to a drug and production of capsular polysaccharide and M proteins were controlled by a unique nucleic acid

The extraction of DNA from the culture of donor pneumococci and its application to recipient pneumococci result in the transformation of some of the recipient organisms The latter acquire a differentiating property of the donor The number of cells transformed is proportional to the amount of DNA applied to a saturation level of up to 10 per cent of all of the treated cells Cells which have been transformed for one property such as penicillin resistance are in general not transformed at the same time for another property such as capsulation Occasional instances of the simultaneous acquisition of two markers are known These results are interpreted as follows The genetic material of the pneumococcus is DNA On extraction it retains its genetic information and, on entering an appropriate host it displaces the homologous genetic material and functions as it did previously in its original host reproducing in synchrony with the cell and causing the expression of some new cellular property Since most of the genes are transformed independently of each other either they never coexisted in the same DNA structure or they were broken apart during the extraction The occasional instances of linked transformation are due to the proximate location on a DNA molecule of the genes controlling these properties

The larger organization of the genetic material in pneumococcus cannot be inferred from transformation As we shall see later

results obtained with other technics indicate that bacterial genetic material is organized in a single linear chromosomelike structure analogous to the organization found in higher forms

In addition to pneumococci transformation has been demonstrated in several other bacterial species These include members of *Hemophilus Bacillus Neisseria Streptococcus* and *Rhizobia* Surprisingly except in a very restricted sense transformation has not been demonstrated in *Enterobacteriaceae* probably a limitation in the reaction is the ability of the DNA macromolecule to penetrate bacteria In all instances of successful transformation there are extremely critical cultural conditions and other factors that are necessary for DNA uptake

The detailed molecular mechanism by which the transforming DNA is incorporated into the genome of the recipient bacterium is still unknown The current view is that the added DNA molecule or a part thereof is used directly by the bacterium and is incorporated into its genetic material for the building of its chromosomes

The work on transformation in bacteria and somewhat similar experiments with bacteriophage focused attention on DNA as genetic material They stimulated efforts to analyze the chemical composition and structure of this molecule

DNA is composed of 2 purine bases adenine and guanine and 2 pyrimidine bases thymine and cytosine the sugar deoxyribose and phosphate Chemical analysis of DNAs from diverse origin and of differing total compositions shows that the molar amount of adenine equals the molar amount of thymine and similarly the molar amounts of guanine and cytosine are equal For each mole of base there is one of sugar and phosphate As prepared by numerous procedures DNA is obviously a large polymer of these basic units

From x ray diffraction patterns of DNA fibers Watson and Crick were able to deduce a structure for DNA molecules This structure is a double helix with 2 intertwined chains of a sugar phosphate backbone the purine and the pyrimidine bases are placed between the chains much as the steps in a ladder The bases in one chain are hydrogen bonded to the bases in the other chain To achieve the required spacings it is necessary for the adenine residues to bond with the thymine residues and the guanine residues to bond with the cystosine residues thus accounting for the molar equivalence of these bases Such a structure is a complementary one such that the sequence of bases in one chain dictates the sequence in the other chain This characteristic immediately suggested a mechanism for DNA replication involving the separation of the chains and the addition of the complementary bases to the separated strands thus maintaining the chemical and therefore the genetic fidelity of the molecule Much evidence has been obtained which supports this mode of replication

The model also implies a mechanism for gene action It is assumed that genes control production of ribonucleic acid (RNA) of a certain type which is then used as a template for the synthesis of protein structures and enzymes RNA being very similar to DNA might be copied from DNA in a manner analogous to that of DNA replication Enzymes which use DNA as a template and synthesize DNA or RNA from nucleotide units have been found in many different organisms

TRANSDUCTION

Transduction was first demonstrated in *S typhimurium* by Zinder and Lederberg Like transformation this process involves the transfer from bacterium to bacterium of bits of genetic material but whereas this material is free in transformation it is carried by a bacterial virus in transduction

There are 2 classes of bacteriophage (bacterial viruses) those that can produce overt infection only (virulent phage) and those that can with varying frequency produce both an overt infection and a latent infection (temperate phage) In the latter instance following infection the phage disappears the bacteria do not produce progeny phage immediately but they now have as a permanent part of their phenotype the potential for the production of phage The nucleic acid of the phage which they carry in a prophage state is reproduced in synchrony

with the rest of the bacterial genetic material

Unlike their more virulent brethren, temperate phages do not kill all of the infected cells therefore alterations of bacterial properties can be seen Following infection with such temperate phages a variable proportion of the infected cells will respond either by lysing or by being latently infected (lysogenized) When the salmonella phage P22 is grown through a vegetative cycle on a well marked strain of *S typhimurium* and is applied to another differentially marked strain there is found among the lysogenic survivors of the infection an alteration in some cells of some of the differentiating properties The gross genetic manifestations of transduction are the same as those in transformation The number of cells transduced for any one property is proportional to the number of phage particles applied Cells transduced for one property are generally not transduced simultaneously for another There is a restriction in the amount of genetic material that can be transduced as the volume available in a phage particle amounts to about 1 per cent of the volume of the bacterial genome

A bacteriophage particle has an outer protein shell and an inner core of the nucleic acid which is its genetic material Apparently during the course of the growth of the phage protein coats are filled by pieces of bacterial nucleic acid instead of phage nucleic acid Such particles occur with fairly low frequency of the order of 1 in 1 million per genetic marker—and probably no more than 1 in 10 000 even if all the bits are summed These then are released with the rest of the phage progeny and on attachment to host bacteria enter them in the same way that phage nucleic acid does Instead of initiating the phage-controlled events this bacterial nucleic acid undergoes the same kind of genetic recombination process with the host genome that the free nucleic acid in transformation does Again instances of linkage are found Both transformation and transduction give rise to situations in which one can study to the exclusion of other genes those genes that are most closely linked together Because transduction was found in salmonella, a wide spectrum of nutritional markers was available for genetic analysis The low frequency of transduction was restrictive only in those instances in which the mutation rate of the gene in question was higher than the transduction frequency of about 10^{-6} *per infecting phage* particle—fortunately a not too frequent occurrence

The ability to concentrate the analysis on linked genes resulted in the revelation of a totally unsuspected feature of the spatial organization of genetic material in bacteria *It also allowed an approach to the funda*mental questions of the fine structure of the gene It was often found that the genes controlling the enzymes of a single biosynthetic pathway were linked We may cite as an instance the genes that control tryptophan biosynthesis There are 4 genes controlling *the synthesis of four enzymes which convert* anthranilic acid to tryptophan, and these are all linked In addition in this instance the order of the genes with respect to each other is the same as that of the reaction sequence Similar findings have been made for other biosynthetic systems This finding has been utilized by Jacob and Monod in developing the operon hypothesis wherein the time and the rate of synthesis of these enzymes are coordinated by their genetic linkage

Since independent mutations could be obtained which caused the loss of the same enzymatic function and therefore presumably were in the same gene it was possible to ask whether these had occurred at the same site in the gene or at different sites If genetic recombination could take place within a gene *even at a low frequency the* enormous resolution provided by the bacterial system could reveal it Phage was grown on one mutant which lacked the enzyme tryptophan synthetase and applied to another independently isolated mutant lacking the same enzyme and the number of the transductions to tryptophan independence was determined If transduction did not occur either the mutations were at the same site or a gene was not divisible by recombination *If transduction did occur the mutations* were at different sites and recombination within a gene could occur Transduction did occur and from the frequency of transduction in such one-by-one pairs there could be deduced a linear order of sites within a gene

which could undergo mutation and recombination The positioning of genes relative to each other is based on the idea common to all genetic recombinational analysis that is the higher the frequency of exchange the farther apart are the genes The same is assumed to be true for sites within a gene Considering the linear nature of the DNA structure this is not an unreasonable assumption Rigid proof of these notions was developed by Benzer in studies of the r gene in the bacteriophage T4

The genetic material that is transduced is not always integrated into the host genome These so-called abortive transductions occur about 10 times more frequently than do stable transductions They were discovered during studies on the transduction of motile flagellated bacteria to nonmotile nonflagellated strains The selection procedure employed a soft agar plate in which nonmotile forms grew but remained fixed at the site of inoculation whereas motile forms swarmed that is to say they migrated through the agar away from the inoculum site In addition to the swarms (stable transductions) there were trails of colonies in the agar proceeding away from the inoculum point These are explained as follows The gene for production of flagella enters a cell and causes the production of flagella For some unknown reason the gene is neither integrated into the genome of the host nor replicated independently of the genome thus it is passed to only one of the progeny cells when the bacterium divides During the period that the cell has flagella it can migrate on loss of the flagella due to segregation of the gene it ceases to migrate and initiates a colony at this point The daughter cell which receives the gene repeats the process hence the row of colonies

Abortive transductions can be found with other genetic markers also When the transduction is from nutritional dependence to independence there are many microcolonies in the agar in addition to the large colonies from the stable transductions These microcolonies are comparable with cells in a trail superimposed one on the other The nonreplicating gene during its passage through a number of cell generations caused the synthesis in each cell of sufficient enzyme to enable the cell to undergo a few further divisions The superposition of these cells forms the microcolony

A number of phages can cause transduction in a number of different bacteria such as *Bacillus Staphylococcus Pseudomonas Proteus* and *E coli* There are many phages which even though temperate do not transduce There is a property of phage growth—probably the way in which it disrupts the bacterial genome—that allows transduction

Transduction of the kind described in the preceding pages is general transduction in that it encompasses the entire bacterial genome with no preference for one gene over another Another variety of transduction also exists This is restricted transduction discovered in *E coli* and its temperate phage lambda When lambda grows in a vegetative cycle it does not cause the transduction of any known bacterial markers when applied to the appropriate host On the other hand when lambda lysogenizes its host and then is caused to undergo a vegetative cycle by induction with ultraviolet irradiation it transduces a few genes affecting the fermentation of galactose and these only The frequency of transduction is of the same order as that in general transduction about 1 per million The transduced cells are unstable Cells transduced from galactose nonfermentation to galactose fermentation continuously segregate galactose nonfermenting cells The frequency is about 1 per thousand per cell generation Apppar ently the transduced fragment is not integrated within the bacterial genome but does replicate almost in perfect synchrony with it Such cells are called heterogenetic They carry their original gene (endogenote) and the transduced fragment the exogenote They are in the older genetic terminology heterozygous but only for a small region of the genome These cells are also lysogenic and carry lambda When they in turn are induced with ultraviolet light they produce an extremely small yield of phage but a very high frequency of transducing particles The detailed analysis of this system has shown that a galactose transducing particle is a phage genome which has exchanged a portion of itself for the bacterium s galactose

with the rest of the bacterial genetic material

Unlike their more virulent brethren temperate phages do not kill all of the infected cells therefore alterations of bacterial properties can be seen Following infection with such temperate phages a variable proportion of the infected cells will respond either by lysing or by being latently infected (lysogenized) When the salmonella phage P22 is grown through a vegetative cycle on a well marked strain of *S typhimurium* and is applied to another differentially marked strain there is found among the lysogenic survivors of the infection an alteration in some cells of some of the differentiating properties The gross genetic manifestations of transduction are the same as those in transformation The number of cells transduced for any one property is proportional to the number of phage particles applied Cells transduced for one property are generally not transduced simultaneously for another There is a restriction in the amount of genetic material that can be transduced as the volume available in a phage particle amounts to about 1 per cent of the volume of the bacterial genome

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The ability to concentrate the analysis on linked genes resulted in the revelation of a totally unsuspected feature of the spatial organization of genetic material in bacteria It also allowed an approach to the fundamental questions of the fine structure of the gene It was often found that the genes controlling the enzymes of a single biosynthetic pathway were linked We may cite as an instance the genes that control tryptophan biosynthesis There are 4 genes controlling the synthesis of four enzymes which convert anthranilic acid to tryptophan and these are all linked In addition in this instance the order of the genes with respect to each other is the same as that of the reaction sequence Similar findings have been made for other biosynthetic systems This finding has been utilized by Jacob and Monod in developing the operon hypothesis wherein the time and the rate of synthesis of these enzymes are coordinated by their genetic linkage

Since independent mutations could be obtained which caused the loss of the same enzymatic function and therefore presumably were in the same gene it was possible to ask whether these had occurred at the same site in the gene or at different sites If genetic recombination could take place within a gene even at a low frequency the enormous resolution provided by the bacterial system could reveal it Phage was grown on one mutant which lacked the enzyme tryptophan synthetase and applied to another independently isolated mutant lacking the same enzyme and the number of the transductions to tryptophan independence was determined If transduction did not occur either the mutations were at the same site or a gene was not divisible by recombination If transduction did occur the mutations were at different sites and recombination within a gene could occur Transduction did occur and from the frequency of transduction in such one by-one pairs there could be deduced a linear order of sites within a gene

was simply to mix 2 lines of *E coli* that had acquired by mutation different nutritional requirements and to seek clones which had no nutritional requirements Thus on an unsupplemented minimal medium only recombinant colonies could grow The limiting frequency that could be measured was determined by the rate of spontaneous mutation of the parental culture to nutritional independence In addition to these selective marker mutations the strains differed by a number of other features which had no gross effect on the ability of the bacteria to grow (unselected markers) These features included fermentative ability drug and phage resistance and nutritional requirements which were supplied in the medium The analysis showed that recombinants were formed at a frequency of about 1 in 10^6 a value well above the frequency of spontaneous mutation in the unmixed parents When these recombinants were further tested for their unselected markers these were found to segregate as if there had been almost complete mixing of the 2 genomes The conclusions from this experiment were (1) bacteria are haploid i e they have only a single representative of each gene (2) they form a complete diploid heterozygote with a low frequency and (3) these zygotes undergo an immediate reduction back to the haploid state with a concurrent process of genetic recombination between the 2 genomes

The selected recombinants represent only one class of the total recombinants formed However because of the low frequency it was necessary to use the selective procedure to find some of them The phenomenon was not contingent on the nature of the selection This was demonstrated by using the same well marked parental cultures but with selection of different sets of markers Depending on the medium employed a given marker could be either selected or unselected The segregation of the unselected markers was not completely random that is it was not a case in which half of the progeny were similar to one parent and half to the other parent The precise pattern for any particular unselected marker depended on the markers that were used for selection of the recombinants This was interpreted as an indication of linkage between the selective and the nonselective markers In the diploid higher forms the genes are located on chromosomes and during the reductive meiotic cycle when the homologous chromosomes pair prior to separation they can undergo recombination with the interchange of parts The amount of interchange between 2 genes is taken as a measure of their physical distance apart on the linear chromosome The closer to one another the 2 genes are on the same chromosome the more rarely do they interchange An analogous mechanism was invoked for bacterial recombination and linkage maps were prepared If a marker had a great tendency to be derived from one of the parents it was presumed to be located near the selected marker of that parent

The original interpretation applied to bacterial mating was that any cell in the bacterial population had an equal probability of mating with any other bacterial cell there was no sexual differentiation From the work of Hayes Cavalli and the Lederbergs this picture was modified to give a simple but precise mating type differential It had been a fortuitous accident that the 2 lines of K 12 that had been used in the majority of crosses had the appropriate mating type

These 2 lines were derived from the original K 12 strain one being a methionine requiring (M—) mutant and the other a triple mutant isolated in sequential steps requiring threonine leucine and thiamine ($T—L—B_1—$) All of the mating stocks of *E coli* have been derived from either of these 2 lines by the addition or the subtraction of genetic markers

Hayes found that although the treatment of the M— line with either streptomycin or ultraviolet light did not prevent recombination treatment of the $T—L—B_1—$ with the same reagents did so Cavalli noted that crosses betwen stocks derived from the $TLB_1—$ line failed whereas crosses between stocks of the M— line still gave recombinants Thus there was a polarity in the mating and the lines were not equivalent in this regard Progeny of a cross could mate with either of the 2 lines In fact culture of the 2 lines together for brief periods followed by reisolation of the TLB_1 line on the basis of some differentiating marker made it possible for this derivative now to mate with

genes The phages are thus incomplete and are called lambda dg for lambda defective carrying galactose genes Such phage particles can grow only in the presence of a complete phage genome A galactose transduced cell contains a complete phage genome and a lambda dg It is the lambda dg that is lost in the unstable transductions

As yet only one other example of restricted transduction has been found that being in *E coli* where the phage ϕ80 carries the region concerned with tryptophan biosynthesis

In addition to acting as a carrier of genetic material from bacterium to bacterium bacteriophages also produce alterations of genetic properties of the host when they are carried as prophages The general property which is conferred on lysogenic organisms in addition to the ability to produce phage at a low, fixed frequency is immunity to superinfection by the phage that is carried

Besides these general alterations of bacterial properties certain bacteriophages produce other changes that are less well understood Perhaps the most profound of these is the conversion of non toxin producing *C diphtheriae* to toxin production by lysogenization with certain bacteriophages The toxin is released during the vegetative growth of the virus either following infection of sensitive cells or during the growth of the lysogenic cells In the latter instance only a small proportion of the cells in each cell generation produce phage and toxin

Phages are also responsible for the presence of certain of the well-characterized somatic antigens of salmonella species Lysogenization by P22 of group B salmonella results in the presence of somatic antigen 1 Those strains that have antigen 1 when isolated from nature can be shown almost invariably to contain P22 or a related phage This conversion is the result of the addition of a terminal glucose residue to the basic polysaccharide structure of the somatic antigen

Another well studied instance of conversion is in Group E of salmonella Strains with antigen 3 10 are modified to antigen 3 15 when they are lysogenized by E 15 phage When the 3 15 strains are further lysogenized by phage E 34, they become 3 15,34 Lysogenization of 3 10 strains by E 34 is without obvious effect The presence of 3 15 is required before E 34 can produce an observable effect Chemical analyses of these antigens reveal the detailed changes underlying these conversions The antigens 3 10 consist partly of a D galactosyl D mannose D rhamnose unit with an alpha linkage between the galactose and the mannose With the conversion to 3,15 this linkage is changed from alpha to beta Phage E 34 causes the addition of a terminal glucose residue to the galactose and apparently the galactose mannose linkage must be beta for this reaction to occur

Other instances of phage conversion are known to occur in other salmonella and in shigella Undoubtedly there are many as yet unresolved similar occurrences in bacteria Lysogenic bacteria are the rule rather than the exception

At present all attempts to develop a logical explanation for these conversions have failed A number of possibilities come to mind The conversions may be accidental in the sense that lysogenization causes an interference with some of the normal metabolic sequences of the cell resulting in these unexpected manifestations Alternatively either the phage may have evolved or the phage and the bacterium evolved from common gene segments the conversion representing the residual activity of this segment A corollary of the latter view is that conversion may represent an early evolutionary experiment in gene transfer Be that as it may it is obviously difficult to decide whether any particular bacterial property is caused by a phage or a bacterial gene In fact this question may be more semantic than real

BACTERIAL CONJUGATION

The mechanisms of genetic exchange that have been described previously are all very restricted in regard to the amount of genetic material that can be transferred at any one time Thus studies employing these mechanisms cannot yield information about the gross organization of bacterial genetic material In 1946 Lederberg and Tatum discovered bacterial conjugation in *E coli* strain K 12 and mutants derived from it The procedure

furthering the analysis The prophage lambda segregated in crosses as if it were linked to the galactose region of the *E coli* chromosome Recall that lambda was involved in the transduction of the galactose region specifically When a lysogenic Hfr was mated with nonlysogenic F— it was found that the prophage on entering the cytoplasm of a nonlysogenic bacterium underwent a process of induction (production of progeny phage) This then could give a precise measure of the zygotic events for it was not contingent on the integration of genetic material The entry of prophage into F— bacteria detected by subsequent phage production coincided in timing with the entrance of the galactose region

Garen and Skaar did a somewhat similar set of experiments with another Hfr (HfrC) but instead of blending they killed the Hfr parent with a virulent bacteriophage Their results also pointed to an orientation of transmission of genetic material but one with a different order than that obtained by Jacob and Wollman for Hfr H Crosses of lysogenic did not give zygotic induction and there was no transmission of the galactose marker

The Hfr have one other property which distinguishes them from F+ cells Mixture of F+ cells with F— cells rapidly converts the latter to F+ but mixture of Hfr with F— is without similar effect While the progeny of an F+ by F— cross are F+ the progeny of Hfr by F— crosses are primarily F— only those few progeny that receive the distal marker of the Hfr become Hfr

Jacob and Wollman developed the following concept of sexual differentiation in *E coli* F— cells lack an agent called F This agent in an F+ cell replicates in synchrony with but independently of the bacterial chromosome and is perhaps responsible for a change in the surface properties of bacteria such that they will unite with F— cells During such a union the F agent itself is transferred with a high frequency but the chromosome with a low frequency The transfer of the chromosome occurs following the same attachment of the F agent to the bacterial chromosome at any of a number of points perhaps at random giving rise to Hfr There can be isolated from F+ cells different Hfr These differ both in the nature of markers which they transmit and in the order of transmission of these markers Jacob and Wollman postulate that the chromosome in either an F— or a F+ cell is a closed ring structure The attachment of the F agent may convert this ring to a linear structure which transfers its chromosome in such a way as to have the F agent terminal

The attachment of the F agent to the bacterial chromosome receives further support from the phenomenon known as F duction Distal markers and the F agent in an Hfr by F— cross are transferred with a low frequency and very late after the mixing of the parental cultures If samples of mating mixtures are blended early after mixing and selection is made for some late entering marker a few recombinants are found These cells have the interesting property of transferring the F agent with a fairly high frequency and in addition to it, a few of the terminal markers of the Hfr which had been used in the original cross The F agent which had been attached terminally to the Hfr chromosome apparently dissociated from the bacterial chromosome carrying with it a bit of bacterial genome A number of different such agents called F′ have been isolated each carrying different bits of bacterial chromosome The genetic markers that are transferred by these contaminated F agents are rarely integrated into the recipient chromosome The situation is somewhat analogous to transduction of the galactose region by the lambda dg Cells carrying such F agents transfer their chromosome with a frequency intermediate between that of an Hfr and an F+ and the transfer has the same orientation as that of the Hfr from which the F agent has been derived The contaminated F agent has a preference for a particular site on the chromosome namely that for which it now has partial genetic homology These F agents are most useful in studying the genetic dominance relationships among bacterial genes as they give rise to persistent heterozygotes for the region involved rather than integrating into the bacterial chromosome

Several agents such as the F′ have been isolated in nature An *S typhosa* lac+ strain

strains with which it was previously incapable of mating. Since genetic recombination occurred at much lower frequencies, it was apparent that fertility itself was being transferred.

The definition of mating type is purely operational. Strains incapable of mating with each other are defined as F—. Strains that can mate with F— strains or with themselves are called F+. Fertility is controlled by the presence or the absence of an agent which is itself transferable in a mating interaction. Cell contact is required for F transfer, as no extraction procedure is successful. Thus conjugation can occur between pairs of bacteria differing primarily in mating type. When the segregation patterns for the unselected markers in these crosses were analyzed, it was found that the majority of the progeny had the markers of the F— parent. Mating type not only influenced the ability to undergo genetic recombination but also had a profound effect on the genetic composition of the progeny.

The interpretation given these results is that in a mating pair there is an unidirectional transfer of genetic material from the F+ strain to the F— strain. The transfer is never complete, thus accounting for the preponderance of markers of the F— parent. The sensitivity of the system itself to agents that kill F— cells but not to agents that kill F+ cells is thereby explained, for it is in reality from the F— cell that the recombinant progeny are derived. The inactivated F+ cells can still transfer some of their genetic material to the viable F— cell. The F agent allows for this genetic transfer.

The proof of this notion depended on a more detailed analysis of the process of mating. This was provided by the discovery of bacteria with a much higher frequency of mating and gene transfer. Two strains (called Hfr strains) were isolated, both derived from the M— line, which when mated with TLB_1— line gave frequencies of recombination of the order of 10 per cent of the parents. The frequency of recombination varied depending on the markers which were used to select the progeny. Apparently there was a gradient with regard to the frequency of transfer of different markers.

Jacob and Wollman studied the kinetics of mating of an Hfr (Hfr H) and F— cells in great detail. They mixed 2 stocks with multiple markers at high bacterial density and then they diluted the mixture at intervals and plated samples in order to determine the recombinant frequency. Other samples were diluted and agitated vigorously in a blendor and then plated for recombinants. Dilution and plating without blending prevented further coupling of bacteria, but those couples that had already formed could still transfer a considerable portion of their genome. However, blending of mated couples separated them at that time and prevented any further transfer of the Hfr genome.

Segregation of the unselected markers was studied in the 2 types of samples. The data were compiled in terms of the per cent of the recombinants that had any particular nonselective marker from the Hfr. For the unblended samples, this fraction remained constant and was independent of the time of sampling. If a marker appeared in 50 per cent of the progeny from the early samples, it had the same frequency in the later ones. However, for the blended samples this fraction increased as a function of the time of sampling, and each marker had its own time of appearance, characteristic rate of increase and maximum. Since the linkage relationship of the markers which was used was known from previous mating experiments, it was possible to analyze the results of the blendor experiment in terms of known linkages. This analysis indicated an oriented sequential passage of markers from the Hfr to the F— in the same linkage relation that had already been inferred.

The mating system described involves (1) combination of the cells of appropriate mating type by a surface interaction, (2) sequential passage of genetic material from Hfr to F—, and (3) its integration into the genetic material of the F—. The limitation in the analysis described above is that it is contingent on the integration of the genetic material. All of the data are obtained from the selected recombinant class, and the mechanism of gene interchange in this system is still incompletely understood and thus might bias the result.

A measurable property of the zygote itself proved to be a most useful tool for

furthering the analysis The prophage lambda segregated in crosses as if it were linked to the galactose region of the *E coli* chromosome Recall that lambda was involved in the transduction of the galactose region specifically When a lysogenic Hfr was mated with nonlysogenic F— it was found that the prophage on entering the cytoplasm of a nonlysogenic bacterium underwent a process of induction (production of progeny phage) This then could give a precise measure of the zygotic events for it was not contingent on the integration of genetic material The entry of prophage into F— bacteria detected by subsequent phage production coincided in timing with the entrance of the galactose region

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Several agents such as the F have been isolated in nature An *S typhosa* lac+ strain

TABLE 1 MATING TYPES OF *E coli*

TYPE	TRANSFERS CHROMOSOME	TRANSFERS F
Hfr	+ + + +	+
F	+ +	+ + + +
F+	+	+ + + +
F—	—	—

was found to transfer the lac character to many different strains including some species of *Proteus* and *Serratia*

Jacob and Wollman have given the name episomes to those elements which have genetic function and can exist in both an autonomous and an integrated state Among these are the temperate phages and the F agents with their varying degrees of contamination

The factors which control the production of colicins also behave as episomes Colicins are antibioticlike agents produced by certain bacterial strains Unlike the other antibiotics they have a limited host range usually only a few species or strains of bacteria are susceptible Fredericq has shown that the capacity to produce colicine can be transferred from strain to strain in a matinglike process However when studied in *E coli* this agent was sometimes transferred independently from the chromosome and at other times as an integrated entity Stocker and Ozeki have shown that the presence of certain colicin agents causes gene transfer in addition to the transfer of the colicin property They mimic the F agent in this respect

A most remarkable episome was discovered in Japan by Akiba About 1953 it was noted that many epidemics of shigellosis were being caused by organisms which exhibited multiple drug resistance The organisms were resistant to streptomycin tetracyline chloroamphenicol and sulfonamides Some patients excreted both sensitive and multiple resistant strains This drug resistance could be transferred in mixed culture from strain to strain through the *Enterobacteriaceae* and to *Serratia* and *Vibrio* strains The agent has been called RTF (resistance transferring factor) Its transfer seems to be quite independent of the F state of the bacteria in fact when RTF and F coexist in the same strain the F induced properties are usually repressed Some of the F— strains carrying RTF can also transfer their chromosomal markers

As with the other autonomous factors the RTF are slowly lost spontaneously during the course of repeated cell culture Hirota has shown that *E coli* can be repeatedly cured of the autonomous F factors by growth in the presence of acridine dyes RTF strains can be similarly cured The acridines apparently affect only the nonintegrated forms as F is not lost from Hfr bacteria when they are similarly treated

The mating types of *E coli* and some of their properties are shown in Table 1

DNA BACTERIAL TAXONOMY AND EVOLUTION

The picture that has emerged of the genetic material of bacteria is a composite based on all of the genetic systems that have been described There is in a bacterium a chromosomelike structure which is composed primarily of DNA There is some evidence both genetic and cytologic that the structure is a closed ring in *E coli* Along the length of the structure in serial fashion are located the genes that control the synthesis of the components of the bacterium The amount of DNA in an *E coli* chromosome is estimated to be 5×10^9 daltons (units of molecular weight) However it is not certain whether the structure represents a single giant molecule of DNA or perhaps smaller units tied together in noncovalent linkages Thus DNA such as that used in transforming systems either is broken in extraction into random pieces or is separated into the discrete molecules There is evidence from transformation that the individual markers reside on definitely characterizable molecules for example different markers have different denaturing temperatures on heating There is also some evidence from transduction that the pieces effecting transmission of any particular marker have fixed not random ends Both results indicate a prepackaging of DNA in definite bits Be that as it may the genes are units of DNA containing on the order of 500 to 1 000 nucleotide pairs The specific sequence of these nucleotide pairs is assumed to be di

rectly related to the amino acid sequence of the secondary gene products the protein structures and enzymes Mutation occurs by the alteration of one or more of the nucleotide pairs within the gene which thereby changes the structure of an enzyme either decreasing or increasing its functional ability Since the gene is fairly large it is apparent that mutation to loss of function is more likely than that to gain of function It is also probable that some base changes may be insignificant for although they cause a change in the structure of an enzyme this change might not alter its active center Such ineffective mutations could accumulate throughout a gene and provide a means for gene evolution

Analysis of the base ratio of the DNA from different bacteria shows that they differ widely in composition, from GC 30 per cent for clostridia to GC 70 per cent for Sarcina (mole % guanine + cytosine/mole % guanine + cytosine + adenine + thymine) From the previous considerations it is apparent that for organisms to be related they should have similar base compositions For example organisms such as coliform salmonella and shigella strains all have about 50 per cent GC While different base ratios can be an indicator of nonrelationship similar base ratios do not prove relationship Many neisseria strains have the same base ratio as organisms of the coliform group Within groups of organisms with the same base ratio more detailed physicochemical analyses of DNA and its sequence can be used as indicators of homologies and hence relationships While the methodology of bacterial genetics makes available some new and scientifically more valid approaches to problems of bacterial taxonomy it seems quite unlikely that these will significantly disturb the current system of classification in the near future

All of the processes of genetic exchange that have been discovered thus far in bacteria involve the partial transfer of genetic material Thus they leave the recipient bacterium intact except for a small bloc of genes In haploid organisms such as bacteria one might imagine that there would be such a degree of homeostasis that the introduction of a large bloc of new genes would produce considerable metabolic unbalance In diploid forms such effects can be cushioned by the homologous chromosome Therefore the systems of exchange offer the organisms the choice of accepting small and perhaps useful bits of genetic material without disturbing their essential integrity There is no doubt that these processes could occur in nature However there is little evidence that they do occur It has been shown that with transformable systems nucleic acid often accumulates during bacterial growth In at least one instance timing of release of nucleic acid by the culture has been demonstrated to coincide with the time of the cells maximum sensitivity to the uptake of nucleic acid Bacteriophages are numerous and of course always are to be found in the same ecologic niche as their hosts so that transductions and conversions could occur The most promiscuous of the sexual like processes F duction has been shown in the laboratory to cross species lines readily Since strains isolated from nature harbor such elements F duction might be quite prevalent We may cite as examples the shigella strains isolated from epidemics in Japan in which multiple drug resistance was shown subsequently to be due to a transferable factor

That genetic exchange plays an important and continuous role in nature cannot be proved or disproved at this time For evolutionary purposes a few instances of gene interchange suffice However it is most obvious that free gene interchange does not occur for if it did we should not expect to encounter the variety of organisms that currently exist The problem of the genetic isolation of microorganisms is in most senses no more complicated than that of the genetic isolation of higher forms Often simple separation due to particular favored ecologic niches separate organisms which are quite capable of gene interchange In time they might evolve in such a way as to prevent interchange even on cohabitation Each of the mechanisms of gene exchange that has been described has obvious limitations in its range and efficiency Transformation reactions involve the uptake of a large linear and highly negatively charged macromolecule For uptake to occur the environmental conditions the strain of bacteria and the stage

of bacterial growth must be critically selected The very high frequencies of transformation that can be achieved in the laboratory require extreme attention to these details Transduction processes and others which involve bacteriophages are restricted to the host range of the phage itself Phages show an exquisite degree of specificity in regard to the organisms to which they can attach For example transducing salmonella phage P22 attaches only to organisms which have somatic antigen 12 Similar restrictions exist for other phages and hence restrict their genetic potential Mating reactions involve the conjugation of 2 bacterial cells and require the presence of particular surface bacterial structures for the building of a fusion bridge In addition to their ability to promote transfer per se transfer factors must also alter the surface of their host bacteria in such a way as to make possible such cellular fusions

Genetic exchange can also be restricted by factors unrelated to the penetration stage While the molecular mechanism of gene recombination is still obscure it is nevertheless apparent that if replacement of material is to occur the exogenote and the endogenote must in some way recognize each other They must have some degree of molecular homology By mating analysis it can be shown that the general order of the genes controlling specific functions is the same in *E coli* and some salmonella Hybrids of *E coli* and salmonella strains have been prepared which have known portions of their genome derived from the salmonella and known portions from the *E coli* By appropriate choice of the hybrid strains can be prepared which will grow the transducing phage P22 Transduction of coli genes by coli genes and transduction of salmonella genes by salmonella genes occurs readily However transduction of coli genes with salmonella genes and salmonella genes with coli genes either fails or occurs with a much reduced frequency In matings in which extensive regions of genetic material are involved sufficient homology can be found for genetic recombination In transduction which involves only small regions there is apparently insufficient homology for the pieces to recognize each other Similar results have been obtained with transformation within various strains of *Hemophilus Bacillus* and *Streptococcus* In the latter instances the gross structure of the genetic material is as yet unknown

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5

Bacterial Metabolism*

In this brief review of bacterial physiology and metabolism no attempt will be made to cover all of the varied patterns found in bacteria and fungi Rather the emphasis will be placed on those aspects which are peculiar to these microorganisms and tend to set them apart from other forms Through such an understanding of differences between microorganisms and their hosts we can hope to develop a more rational approach to chemotherapy and to other means for the control of bacterial infection However we must also understand the ways in which bacteria differ in their metabolic patterns from each other since these differences frequently provide the means for distinguishing between closely related species Together with morphologic and immunologic criteria metabolic characteristics provide a basis for the classification of bacteria Furthermore the presence or the absence of a metabolic trait frequently may be correlated with the presence or the absence of pathogenicity

* Abbreviations used in this chapter include the following
AMP—adenosine monophosphate
ATP—adenosine triphosphate
CDP—cytidine diphosphate
DNA—deoxyribonucleic acid
DNP—dinitrophenol
DPN—diphosphopyridine nucleotide
DPNH—reduced diphosphopyridine nucleotide
GTP—guanosine triphosphate
IDP—inosine diphosphate
ITP—inosine triphosphate
RNA—ribonucleic acid
TPN—triphosphopyridine nucleotide
TPNH—reduced triphosphopyridine nucleotide
UDP—uridine diphosphate

GROWTH

Methods of Measurement

In any population of cells growth may result either from an increase in the mass of the individual cells or from an increase in the cell number Under ideal conditions the average cell size or mass is very constant and a determination of one represents a determination of the other Thus under true physiologic conditions procedures which measure either the total number of cells or the total amount of cell material will provide suitable indices of growth The total number of cells may be counted directly using a counting chamber particularly with dark field illumination More frequently the number of viable cells in the population is determined by plating the cells on suitable growth media and counting the colonies which arise from the individual cells The latter procedure yields the viable count since it does not include cells which are dead and fail to give rise to colonies Growth may also be determined by measuring the bacterial density in one of several ways The basic reference is provided by measurement of the dry weight which can be related to nitrogen content metabolic activity packed cell volume or most common of all turbidity or absorbancy of light (optical density) at a prescribed wave length

The data obtained ln any of these pro cedures may be expressed by plotting the appropriate parameter against time When cells which have not been growing actively (usually referred to as resting cells) are placed in a medium which can support growth the growth curve passes through several phases shown schematically in Figure 1 *In the first phase called the lag phase* there is usually very little increase in cell number although there may be some increase in cell mass This is followed by the loga rithmic or exponential phase in which the cells are increasing in mass and dividing at an exponential rate Ultimatelj if the cells are not transferred to *fresh medium they* will pass into the resting or stationary phase during which there is little further increase in mass or cell number In many cases this *is followed* by a decrease in the number of cells They will pass into the death phase or phase of decline in which there may be a breakdown of cells or lysis and a liberation of their contents into the medium

Since bacteria divide by a process called binary fission their number (B) will increase by powers of two,

$$B = b\,2^{n}$$

where b is the initial number of cells and n the number of cell divisions or generations This equation is an expression of the growth rate during the exponential phase For the time interval $t\ t_0$ (in hours) this may be expressed as

$$B = b\,2^{(t\ t)}$$

where r is the growth rate in divisions per hour, therefore $n = r(t\ t_0)$ This equation can be converted to the logarithmic form

$$\log_{.} B - \log\ b = r(t\ t\ )$$

The gcneration time g is the reciprocal of *the growth rate r and is therefore equal to*

$$g = \frac{t\ t}{(\log\ B - \log\ b)}$$

Often it may be more convenient to express this in ordinary logarithms

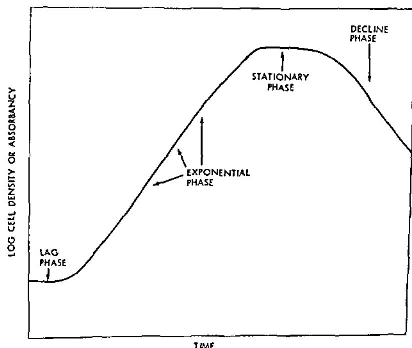


FIG 1 Bacterial density curve showing phases of growth

$$g = \frac{t\ t}{0\ 301(\log_{10} B - \log_{10} b)}$$

The generation time can be read from a plot of log B versus t If the data are plotted in ordinary logarithms (base 10) then the time required for a change of 0 301 represents the generation or doubling time

FACTORS AFFECTING THE GROWTH RATE

During the lag phase the failure of the cells to divide may reflect the time required to fill their metabolic pools and synthesize the appropriate enzymes and coenzymes During the exponential phase the growth rate may vary according to the composition of the medium the temperature and the rate of aeration With aerobic cells vigorous stirring and aeration frequently with pure oxygen may be required for maximum growth rates The nature of the substrate and the composition of the medium will affect growth In general cells grown on enriched medium will divide more frequently than cells growing on a minimal medium Reasons for this will be discussed in a later section

The effect of temperature on growth rate may be quite complex In the ordinary temperature range the growth rate usually will increase with temperature much as do physiologic processes in general At higher temperatures growth may slow down or cease Optimum temperature for growth is usually a reflection of the normal environment of the organism Thus pathogenic bacteria usually will grow best at 37 C Yeasts and fungi for the most part prefer lower temperatures and are usually cultivated at room temperature or 30 C Bacterial cells vary enormously in their response to elevated temperatures and in general pathogenic species are inhibited in their growth or even killed at temperatures above 45 C Occasionally mutations are encountered which result in changes in thermostability or thermolability In nature we find organisms growing at temperatures approaching the boiling point of water These are generally referred to as thermophils and will grow at temperatures at which other organisms are unable to grow The biochemical basis for differences in response to temperature remains obscure

Cells entering the stationary phase of growth may do so because of changes in the environment such as a fall in pH exhaustion of some essential component from the

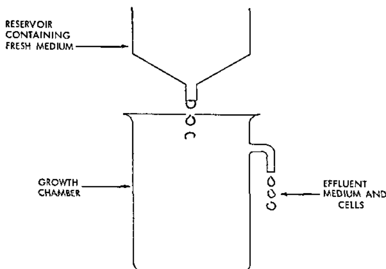


FIG 2 Diagrammatic representation of chemostat for controlled exponential growth of cells

medium, or accumulation of a toxic metabolite Aerobic organisms may cease to grow when they are crowded simply because it is impossible to provide enough oxygen even by vigorous aeration to maintain further growth However if such changes in the medium are avoided the cells may be maintained in the exponential phase indefinitely An example of this is provided by the use of the chemostat This is a device in which fresh medium is continually added to the cell suspension at the same rate at which medium and cells are removed from the system Under these conditions the number of cells will increase at first until one of the components of the growth medium becomes limiting then the cells will grow at a rate which is determined by the rate of addition of this component This apparatus is represented diagrammatically in Figure 2 The chemostat is of value in studies where it is desired to have a predetermined generation time and yet maintain exponential growth conditions It is also used to determine effects of added metabolites since in the chemostat the limiting metabolite generally exists at very low concentration

Synchronous Growth

Under appropriate conditions a population of cells will divide synchronously yielding discontinuous growth curves based on viable count data Cells may be brought into synchronous growth by allowing an essential component of the medium to be depleted and then adding this component or by abrupt changes in temperature Perhaps the most physiologic method for bringing cells into synchronous growth is to use an inoculum of young cells which can be obtained by selective filtration The smallest cells which pass through appropriate selective filters will be growing cells which have just undergone cell division for a few generations these will continue to grow and divide synchronously Such cell populations are useful in the study of control mechanisms which regulate cell growth and division

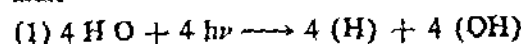
Nutritional Requirements for the Growth of Bacteria

A suitable environment which will support the growth of a microorganism must contain sources of all of the elements which go to make up the bacterial cell The principle elements required are carbon nitrogen phosphorus, sulfur oxygen and hydrogen In addition most cells require significant quantities of a number of cations including potassium manganese magnesium and iron as well as calcium zinc and copper Trace quantities of a number of other cations are found in bacterial cells but these are usually introduced as contaminants of other components of the medium and it is difficult to demonstrate a definitive need for these compounds The principle anions required for growth are chloride sulfate and phosphate Some cells are unable to utilize sulfate and sulfur and for these sulfur must be provided in the form of organic or inorganic sulfides Finally all cells in order to grow must have available a suitable source of energy Often this is furnished by the same compound which provides the source of carbon

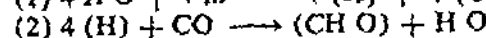
Although bacteria are similar in their basic composition they differ in synthetic capacity Many species are able to survive on purely inorganic media and to derive carbon from carbon dioxide These are designated *lithotrophs* These organisms must use either light or chemical energy to accomplish the reduction of carbon dioxide by the elements of water The *photosynthetic lithotrophs* gain their energy from light by the process of photosynthesis the *chemosynthetic lithotrophs* obtain energy by the oxidation of inorganic substances such as ferrous iron sulfide elemental sulfur or thiosulfate ammonia nitrite, or even hydrogen

Photosynthesis in bacteria is unlike photosynthesis in higher plants in one important respect it is never accompanied by the production of oxygen Photosynthetic bacteria generally thrive only in an anaerobic environment which also contains a suitable hydrogen donor or reducing agent This difference between photosynthetic bacteria and green plants is illustrated best by a consideration of the equations of photosynthesis In green plants the process is represented by the following equations in which $h\nu$ represents the energy in a quantum of light (H) and (OH) represent reducing and oxidizing products derived from water and (CH_2O)

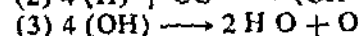
represents the ultimate product carbohydrate



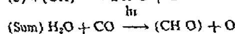
$$(1)\ 4\,H_2O + 4\,h\nu \longrightarrow 4\,(H) + 4\,(OH)$$



$$(2)\ 4\,(H) + CO_2 \longrightarrow (CH_2O) + H_2O$$

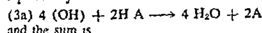


$$(3)\ 4\,(OH) \longrightarrow 2\,H_2O + O_2$$



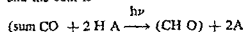
$$(\text{Sum})\ H_2O + CO_2 \xrightarrow{h\nu} (CH_2O) + O_2$$

In photosynthetic bacteria the reaction represented by equation 3 (the production of oxygen) does not occur this reaction is replaced by



$$(3a)\ 4\,(OH) + 2H_2A \longrightarrow 4\,H_2O + 2A$$

and the sum is



$$(\text{sum})\ CO_2 + 2\,H_2A \xrightarrow{h\nu} (CH_2O) + 2A$$

H_2A represents a hydrogen donor or reducing agent this may be hydrogen sulfide or another inorganic reducing agent or in some species an organic reducing agent such as malic acid In the photosynthetic sulfur bacteria large quantities of free sulfur accumulate The nutritional requirements of photosynthetic organisms will be determined by the nature of the reducing agent which they can employ The situation is very similar for chemosynthetic organisms

In contrast with the lithotrophs the *organotrophs* (or *heterotrophs*) require as a source of energy an organic nutrient which they can oxidize or ferment The photosynthetic organisms which require organic reducing compounds are also classified as organotrophs more specifically they are called *photo-organotrophs* or *photoheterotrophs* In addition bacteria may require a variety of organic growth factors including many of the vitamins which are required by man and often in addition certain amino acids purines pyrimidines and other substances Many species may have very complex growth requirements and some pathogens cannot be cultivated on defined media Microorganisms which require specific growth factors have been widely employed as sensitive microbiologic assay systems since under defined conditions the amount of growth is directly proportional to the quantity of growth factor provided Before the advent of chromatographic methods microbiologic assays were common for amino acids purines and pyrimidines It was with the aid of such methods that it was established that deoxyribonucleic acids contain equivalent amounts of adenine and thymine and of guanine and cytosine

In recent years microbiologic assays have been used for the isolation of new growth factors some of which have subsequently been found to be important in the nutrition of higher animals Often bacteria require more complex forms of the vitamins than are needed in animal nutrition For example *Hemophilus influenzae* will not grow on nicotinic acid or nicotinamide but requires the intact nicotinamide riboside linkage and will grow best on DPN or TPN Often these requirements for more complex forms of the vitamin have provided clues to the nature of the coenzyme form An example of this is the structure of coenzyme A which contains pantothenic acid The vitamin pantothenic acid was first shown to promote yeast growth and later to be required for many bacterial species The 2 components of pantothenic acid β alanine and pantoic acid will replace pantothenic acid itself in some cases since some species cannot synthesize the β alanine portion while others cannot synthesize pantoic acid *Lactobacillus bulgaricus* on the other hand requires a conjugated form of pantothenic acid pantotheine This substance at one time called the lactobacillus factor can be derived from coenzyme A by the action of phosphatase and its identification was of great importance in the elucidation of the structure of coenzyme A The arrangement of these components in the coenzyme A molecule is shown in Figure 3

Bacterial vitamins include many of the vitamin B group such as thiamine riboflavin pyridoxal and nicotinic acid In addition bacteria may also require p amino benzoic acid folic acid hemin and vitamin K Sterol vitamins are unknown and the presence of sterols in bacteria has not been substantiated although certain yeasts may contain large quantities Nor has any requirement for ascorbic acid been demonstrated in bacteria Carotenes participate in bacterial photosynthesis

Origin of Nutritional Requirements

It is of interest to consider the possible evolution of these growth requirements and

medium or accumulation of a toxic metabolite Aerobic organisms may cease to grow when they are crowded simply because it is impossible to provide enough oxygen, even by vigorous aeration to maintain further growth However if such changes in the medium are avoided the cells may be maintained in the exponential phase indefinitely An example of this is provided by the use of the chemostat This is a device in which fresh medium is continually added to the cell suspension at the same rate at which medium and cells are removed from the system Under these conditions the number of cells will increase at first until one of the components of the growth medium becomes limiting then the cells will grow at a rate which is determined by the rate of addition of this component This apparatus is represented diagrammatically in Figure 2 The chemostat is of value in studies where it is desired to have a predetermined generation time and yet maintain exponential growth conditions It is also used to determine effects of added metabolites since in the chemostat the limiting metabolite generally exists at very low concentration

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for energy *Aerobes* depend on respiration and require oxygen these must be grown with shaking or aeration *Faculative anaerobes* can derive energy from fermentation and will grow in the absence of oxygen but will survive and often thrive in the presence of air *Obligate anaerobes* on the other hand can be grown successfully only in the complete absence of oxygen and may be killed if even traces of air are present

Electron Transport in Aerobic Microorganisms

Aerobic organisms derive their energy from oxidations which resemble those found in higher animals This process known as *respiration* involves the oxidation of the substrate by molecular oxygen through the intervention of the electron transport mechanism (Fig 4) The bacterial cytochromes which catalyze the terminal steps in the chain of electron transport (Fig 5) resemble those found in mammalian cells and in yeast and studies of absorption spectra of bacteria have disclosed the presence of cytochromes of the a a_3 b c and c_1 types However bacterial cytochromes frequently differ from mammalian or yeast cytochromes in the precise location of their absorption bands as well as in their catalytic properties In general cytochrome of the c type isolated from bacteria will not react with reduced pyridine nucleotide in the presence of mammalian cytochrome reductase and the oxidation of the reduced form is not catalyzed by mammalian cytochrome oxidase (cytochromes a + a_3) Some species of microorganisms are completely lacking in cytochrome c yet possess an active electron transport chain Cytochromes of the c type also function in bacterial photosynthesis and in nitrogen fixation

Cytochromes of the b type are frequently encountered in bacteria and may act as components of the electron transport mechanism as is shown in Figure 5 In a few cases they have a distinctly different function Lactic dehydrogenase isolated from yeast has been characterized as a cytochrome b like compound (designated cytochrome b) and a cytochrome of this type has also been im

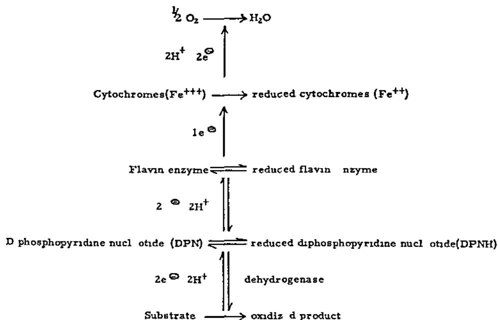


Fig 4 Schematic representation of the path of electrons and protons in respiration

particularly to inquire as to the evolutionary sequence of appearance of lithotrophs and organotrophs According to an older theory primitive organisms possessed very simple growth or nutritional requirements According to this view heterotrophic forms developed through a gradual loss of synthetic power by a type of functional degeneration Thus mutants with specific deletions would be forced to satisfy their requirements from the surrounding medium This theory was attractive because such changes (loss of synthetic capacity) were commonly observed in spontaneous or induced mutants Recently however there has been a tendency to favor the opposite point of view namely that the most primitive organisms were organotrophs which arose in primordial seas containing all of the requirements of the cell These organisms would be expected to possess a very limited synthetic capacity As essential nutrients were depleted from the environment mutants were selected which had developed the capacity to synthesize the missing precursors The attractive feature of this second theory is that it avoids the requirement for a sudden appearance of a living cell having a very complex metabolic machinery capable of synthesizing all of the essential components of the cell This hypothesis is also in accord with current concepts as to the composition of the primordial hydrosphere

Changes in Cell Composition With Changes in Growth Rate

It has been mentioned that the growth rate (or reciprocal of the generation time) is a function of the composition of the medium and that cells generally will grow more rapidly in an enriched medium where they are not required to synthesize all of the amino acids purines pyrimidines and other cell components Such rapidly growing cells also show increases in average cell size and in the number of nuclei per cell They are relatively rich in their complement of RNA DNA and protein although these increases are not proportionate Thus bacteria isolated from enriched medium contain relatively more RNA and protein than DNA as compared with cells growing in minimal medium This effect is seen most strikingly when cells are transferred from minimal to enriched medium the quantity of DNA and total mass per cell may increase by approximately 2 fold while the content of RNA and protein may increase as much as 3 fold These changes follow a particular time sequence When cells growing exponentially in a minimal salt medium are transferred to nutrient broth several generations may pass without any notable change in the rate of cell division The first change to be observed is an immediate increase in the rate of synthesis of RNA This is followed later by a change in the rate of synthesis of protein and finally of DNA Ultimately after about 60 minutes have elapsed there will also be observed an increase in the rate of cell division These changes may be related to increases in specific types of RNA and to cellular control mechanisms (see below)

ENERGY METABOLISM

Bacteria fall into several groups according to their means of satisfying their requirement

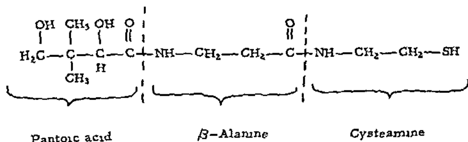


Fig 3 Structure of pantotheine and its components In Coenzyme A adenosine 3 phosphate is attached to the terminal OH group of pantoic acid by a 5 pyrophosphate bridge

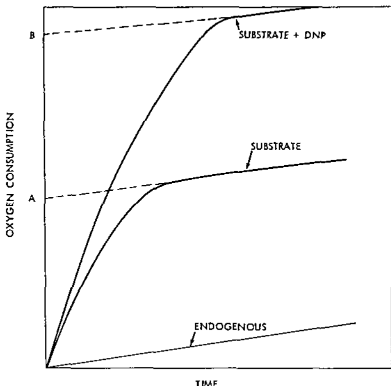


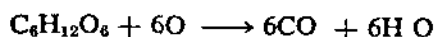
FIG 6 Schematic representation of oxidative assimilation and its uncoupling by dinitrophenol A and B represent the net oxygen uptake due to the substrate in the absence and the *presence of dinitrophenol* respectively

cultivated in the presence of oxygen if agents are added to maintain a low oxidation reduction potential Examples of such agents are ascorbic acid and cysteine both of which will facilitate growth of obligate anaerobes in the presence of oxygen It is noteworthy that these compounds will reduce oxygen to hydrogen peroxide and therefore tend to increase rather than decrease the concentration of this substance

A better hypothesis to account for the sensitivity of obligate anaerobes to oxygen is based on the sensitivity to oxidation of the proteins found in anaerobes Enzyme preparations from clostridia must be protected from oxygen by maintaining anaerobic conditions or by the addition of sulfhydryl compounds such as mercaptoethanol or thioglycolate The proteins of these organisms are peculiarly sensitive to inactivation by oxidation This may be a reflection of their primitive evolutionary state In higher aerobic species these protein SH— groups are folded into the center of the molecule and thus are protected from oxidation They are exposed when the protein is denatured

OXIDATIVE ASSIMILATION

Aerobic organisms oxidize only a portion of the substrate which they consume the remainder is converted to cell material In the presence of a limiting amount of a carbon (and energy) source oxygen consumption continues until all of the substrate is depleted from the medium following which it returns to the low endogenous level characteristic of resting cells (Fig 6) Calculation of the amount of oxygen consumed relative to the amount of substrate added generally will show that about half the substrate is oxidized the remainder of *substrate utilization is not accounted for* either by carbon dioxide production or oxygen consumption As an example in the case of glucose the equation for respiration



$$C_6H_{12}O_6 + 6O \longrightarrow 6CO + 6H\ O$$

requires the uptake of 6 moles of O for each mole of glucose consumed With most aerobic organisms the observed value will be closer to 3 than to 6 Oxidative assimilation of substrate is common to a wide

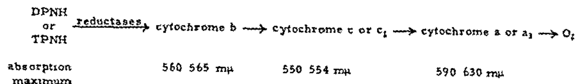


FIG 5 Role of the cytochromes in electron transport and their characteristic absorption maximum

plicated in succinate oxidation in *Coryne bacterium diptheriae*

In general very little is known about the precise function of the bacterial cytochromes Their role in respiration is evident from the fact that in most bacterial species which contain the cytochrome components respiration is inhibited by cyanide carbon monoxide or azide reagents which are known to block the functioning of the cytochrome system Bacterial cytochromes also participate in two other important physiologic functions namely nitrogen fixation and photosynthesis *Azotobacter* species which can carry out nitrogen fixation are among the richest sources of cytochromes Isolation and characterization of these pigments has been hampered by the difficulties in obtaining soluble preparations

The terminal oxidation system of bacteria resembles that of mammalian cells in its coupling to oxidative phosphorylation Respiratory particles capable of carrying out coupled oxidative phosphorylation have been isolated from many species of bacteria The P/O ratio obtained with these systems is usually less than with mammalian preparations but there is little doubt that this is a major energy yielding mechanism in aerobic microorganisms Like the mammalian system bacterial oxidative phosphorylation is uncoupled by dinitrophenol

The respiratory enzymes of bacteria appear to be associated with the cell membrane While there is no evidence for structures like the mitochondria of mammalian cells the chemical composition of the membrane which is rich in lipoprotein resembles that of the mammalian mitochondrial particle

The Flavin Enzymes

Many species of bacteria exhibit respiration which is not sensitive to carbon monoxide or cyanide Such species usually can be shown to contain considerable quantities of flavin and it is likely that their oxygen consumption is due to the action of flavoprotein enzymes Examples of microorganisms which show this type of respiration include the lactobacilli and the pneumococcus When these organisms are grown in the presence of air they produce hydrogen peroxide which can be detected either by its action on hemoglobin which it converts to a green pigment or by the benzidine peroxidase test (used for pneumococcus) These organisms are generally classified as faculative anaerobes and it is questionable whether their aerobic metabolism is of physiologic importance

Bacteria which lack cytochromes are generally also lacking in catalase and therefore are unable to remove hydrogen peroxide which is produced under aerobic conditions

Terminal Respiration Systems in Obligate Anaerobes

Many species of bacteria including species of clostridia, will when placed under aerobic conditions take up oxygen with the production of hydrogen peroxide but are either killed or inhibited from growth under these conditions It has been suggested that they are killed by the hydrogen peroxide which they produce since they lack catalase Two important objections may be raised to this hypothesis In the first place many species which lack catalase such as pneumococcus will continue to grow in air despite the accumulation of hydrogen peroxide Furthermore it has been established that the obligate anaerobes are not killed by oxygen itself nor by a product of oxygen metabolism but rather by the high oxidation reduction potential which results from the presence of oxygen Even obligate anaerobes can be

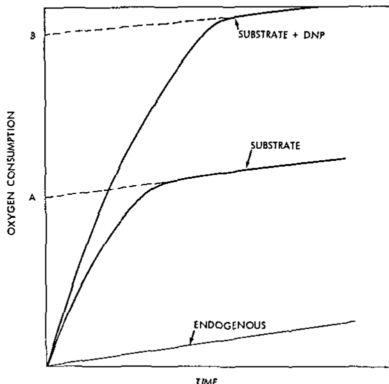


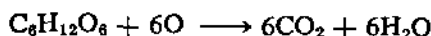
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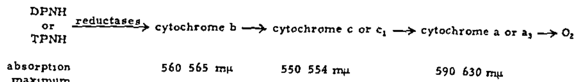


FIG 5 Role of the cytochromes in electron transport and their characteristic absorption maximum

plicated in succinate oxidation in *Coryne bacterium diptheriae*

In general very little is known about the precise function of the bacterial cytochromes Their role in respiration is evident from the fact that in most bacterial species which contain the cytochrome components respiration is inhibited by cyanide carbon monoxide or azide reagents which are known to block the functioning of the cytochrome system Bacterial cytochromes also participate in two other important physiologic functions namely nitrogen fixation and photosynthesis *Azotobacter* species which can carry out nitrogen fixation are among the richest sources of cytochromes Isolation and characterization of these pigments has been hampered by the difficulties in obtaining soluble preparations

The terminal oxidation system of bacteria resembles that of mammalian cells in its coupling to oxidative phosphorylation Respiratory particles capable of carrying out coupled oxidative phosphorylation have been isolated from many species of bacteria The P/O ratio obtained with these systems is usually less than with mammalian preparations but there is little doubt that this is a major energy yielding mechanism in aerobic microorganisms Like the mammalian system bacterial oxidative phosphorylation is uncoupled by dinitrophenol

The respiratory enzymes of bacteria appear to be associated with the cell membrane While there is no evidence for structures like the mitochondria of mammalian cells, the chemical composition of the membrane which is rich in lipoprotein resembles that of the mammalian mitochondrial particle

The Flavin Enzymes

Many species of bacteria exhibit respiration which is not sensitive to carbon monoxide or cyanide Such species usually can be shown to contain considerable quantities of flavin and it is likely that their oxygen consumption is due to the action of flavoprotein enzymes Examples of microorganisms which show this type of respiration include the lactobacilli and the pneumococcus When these organisms are grown in the presence of air they produce hydrogen peroxide which can be detected either by its action on hemoglobin which it converts to a green pigment or by the benzidine peroxidase test (used for pneumococcus) These organisms are generally classified as faculative anaerobes and it is questionable whether their aerobic metabolism is of physiologic importance

Bacteria which lack cytochromes are generally also lacking in catalase and therefore are unable to remove hydrogen peroxide which is produced under aerobic conditions

Terminal Respiration Systems in Obligate Anaerobes

Many species of bacteria including species of clostridia, will when placed under aerobic conditions take up oxygen with the production of hydrogen peroxide but are either *killed or inhibited from growth under these conditions* It has been suggested that they are killed by the hydrogen peroxide which they produce since they lack catalase Two important objections may be raised to this hypothesis In the first place many species which lack catalase such as pneumococcus will continue to grow in air despite the accumulation of hydrogen peroxide Furthermore it has been established that the obligate anaerobes are not killed by oxygen itself nor by a product of oxygen metabolism but rather by the high oxidation reduction potential which results from the presence of oxygen Even obligate anaerobes can be

storage products resembling glycogen In many microorganisms the storage product is a polymer of β hydroxybutyric acid These polymers accumulate during the oxidation of glucose in the absence of nitrogen and account quantitatively for the substrate which is not oxidized When the cells are returned to medium containing nitrogen the storage products are consumed and converted to protein and other cell components

PATHWAYS OF CARBOHYDRATE METABOLISM

The Embden Meyerhof Pathway and the Citric Acid Cycle

Most bacteria oxidize substrates by pathways similar to those found in higher animals Glucose is metabolized by the Embden Meyerhof pathway (Fig 7) and the end product pyruvic acid is oxidized by the citric acid cycle (Fig 8) In most aerobic bacteria the citric acid cycle serves a dual function (1) it provides energy through the oxidation of reduced pyridine nucleotides by way of the cytochrome electron transport mechanism and (2) it provides precursors for the synthesis of amino acids Thus as is shown in Figure 8 arginine proline and lysine are derived from α ketoglutarate while aspartic acid isoleucine homoserine threonine and methionine are derived directly or indirectly from oxalacetate In bacteria lysine is derived from oxalacetate via diaminopimelic acid while in fungi it is produced from glutamate by way of α amino

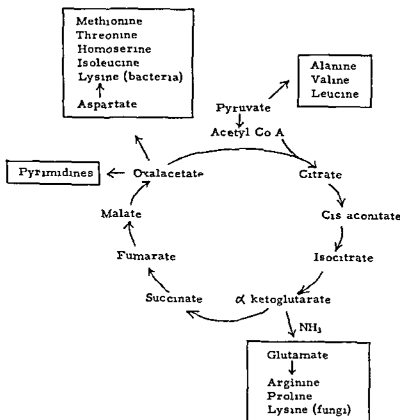


Fig 8 Origin of the amino acids and pyrimidines from citric acid cycle intermediates Primary fixation of NH_3 occurs only in the formation of glutamic acid from α ketoglutarate all other amino acids are formed via transamination reactions

variety of aerobic bacteria and substrates and can be blocked by dinitrophenol (DNP) This agent usually increases the initial rate of oxygen consumption but more striking is the fact that the total oxygen consumed is greater and in general will reach the theoretical value for complete oxidation This effect of DNP as an uncoupling agent for assimilation is analagous to its uncoupling of oxidative phosphorylation in mammalian cells The discovery of this effect of DNP in the uncoupling of assimilation in bacteria antedates its introduction as a tool for the study of oxidative phosphorlyation in mammalian cells

Assimilation of substrate by bacteria is a complex process Under growth conditions the assimilated substrate is converted to normal cell constituents protein lipid and nucleic acid However it does not follow that cells prevented from growth are unable to assimilate substrate Thus, in the absence of a source of nitrogen glucose and other substrates yield only about 50 per cent of the theoretical oxygen consumption the remainder of the substrate carbon is assimilated The most common products of substrate assimilation are polymers such as cellulose or a variety of capsular polysaccharides Bacteria commonly accumulate internal

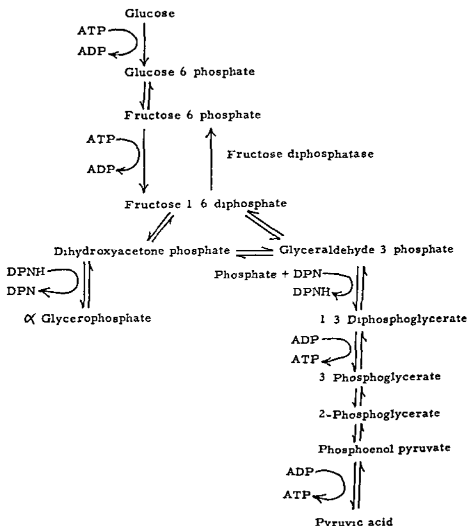


FIG 7 The Embden Meyerhof or Glycolytic Pathway

which can be assimilated via phosphoenol pyruvate Cells adapted to growth on acetate also contain a very active fructose 1 6 diphosphatase which permits the synthesis of hexose by a reversal of the glycolytic pathway (Fig 7)

Alternate Pathways of Carbohydrate Metabolism

Not all microorganisms metabolize carbo hydrate via the Embden Meyerhof pathway Deviations from this common pattern are frequently encountered in anaerobes and also in aerobic species carbohydrate metab olism may involve other pathways In this section we shall consider some of these alternate aerobic pathways

The pentose phosphate pathway, or hexo semonophosphate shunt, is present in nearly all species of bacteria whether or not they also contain the Embden Meyerhof pathway This pathway branches off from the Embden Meyerhof pathway at the level of fructose 6 phosphate which thus serves as a site for the action of cellular control mechanisms (Fig 10) Glucose 6 phosphate and fruc tose 6 phosphate are converted to pentose phosphate by one of two possible mech anisms In the first glucose 6 phosphate is oxidized directly at the C-1 position to yield 6 phosphogluconate which in turn is oxi dized to ribulose 5 phosphate The coenzyme for this oxidation is usually triphosphopyri dine nucleotide (TPN) although in some species of bacteria it is replaced by diphos phopyridine nucleotide (DPN) The primary product ribulose 5 phosphate is in equilib rium with ribose 5 phosphate the precursor of nucleic acid ribose or with xylulose 5 phosphate Xylulose 5 phosphate and ribose 5 phosphate are converted to sedoheptulose 7 phosphate and triose phosphate which in turn react to yield fructose 6 phosphate and erythrose 4 phosphate Finally to complete the cycle erythrose 4 phosphate can react with a second molecule of xylulose 5 phos phate to yield fructose 6 phosphate and triose phosphate In the oxidation of 6 phos phogluconate to ribulose 5 phosphate 1 mole of carbon dioxide is produced thus it is theoretically possible to pass a hexose phosphate molecule around the cycle 6 times and thereby to oxidize it completely to carbon dioxide and water It is to be noted that this series of reactions produces TPNH which unlike DPNH is not a substrate for the electron transport pathway and does not yield energy through oxidative phosphoryla tion None of the reactions of the pentose phosphate pathway will generate ATP and

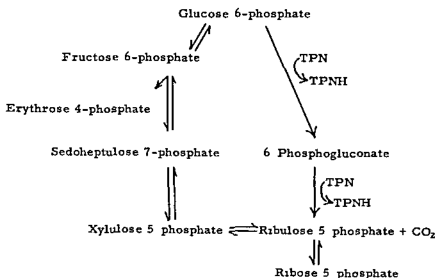


Fig 10 The Pentose Phosphate Pathway

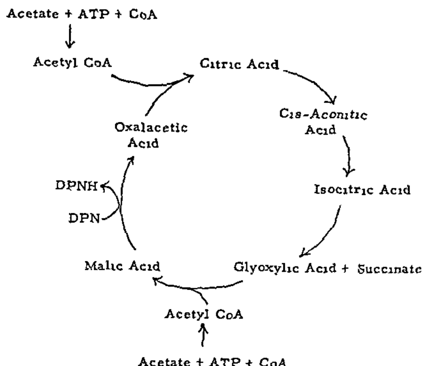
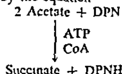
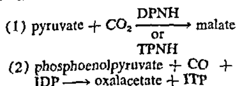


Fig 9 Conversion of acetate to succinate by the glyoxylate by pass The over all result may be expressed by the equation



adipic acid Pyruvate serves as a precursor of alanine valine and leucine In the absence *of amino acid or pyrimidine synthesis the* citric acid cycle is self sustaining with each turn of the cycle one equivalent of pyruvate is oxidized to yield 3 equivalents of carbon dioxide Oxalacetate is consumed in the formation of citrate and is regenerated in equivalent quantities However the removal of α ketoglutarate or oxalacetate for amino acid synthesis changes the character of the cycle since it can no longer sustain itself *when the pool of dicarboxylic acids* is depleted In order to maintain the cycle dicarboxylic acids must constantly be produced by other reactions Two such reactions are known



(1) pyruvate + $CO_2 \xrightarrow[\text{TPNH}]{\text{DPNH or}}$ malate

(2) phosphoenolpyruvate + CO + IDP $\longrightarrow$ oxalacetate + ITP

The first of these reactions catalyzed by the malic enzyme has been found in bacteria Thus far, the second carboxylase has not been detected Most of the CO produced in the citric acid cycle must be returned for the regeneration of dicarboxylic acids and it may be concluded that in bacteria the primary *function of the cycle is the* biosynthesis of amino acids rather than as a mechanism for the oxidation of pyruvate

The Glyoxylate Bypass

A special case exists in organisms using acetate for growth Theoretically acetate can *be converted to isocitrate by way of* acetyl CoA and the condensation reaction with oxalacetate However while it can be oxidized completely *by this series of reactions* it is impossible for it to be utilized for assimilation or biosynthesis Organisms which *grow on acetate as a sole carbon source* possess a modified citric acid cycle known as the glyoxylate bypass (Fig 9) Acetyl CoA condenses with oxalacetate *in the usual* way to form citric acid However isocitric acid is not oxidized to α ketoglutarate as in the citric acid cycle but is split in an aldolase type reaction to form glyoxylate and succinate Glyoxylate condenses with the second molecule of acetyl CoA to form malic acid which is converted to oxalacetic acid to complete the cycle This process provides a means for the net synthesis of C_4 acids

citric acid cycle For example citrate will support the growth of *Aerobacter aerogenes* but will not serve as a growth substrate for the closely related organism *Escherichia coli* The basis for this difference has been shown to be one of permeability *E coli* metabolizes internally produced citrate but is unable to take it up from the medium

In organisms lacking the citric acid cycle other mechanisms largely unknown are responsible for the synthesis of amino acids

Partial Oxidations

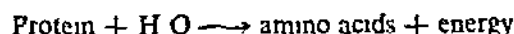
Many species of microorganisms derive energy by carrying out partial oxidations A classic example is the microorganism which catalyzes the oxidation of ethanol to acetic acid and was studied by Pasteur in his efforts to determine the cause of spoilage of wine Later Bertrand discovered the oxidation of polyols to ketoses by acetobacter a process which has been widely used by synthetic organic chemists for specific syntheses such as the formation of sorbose from sorbitol This is also the basis for the industrial production of gluconate and 2 ketogluconate These partial oxidations are accomplished by particulate enzymes which are coupled to energy production by unknown mechanisms In these organisms only a small fraction of the substrate is assimilated in this respect the processes resemble fermentation rather than respiration They probably represent a primitive respiration mechanism

FERMENTATION MECHANISMS

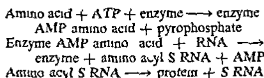
The Problem of Energy Production ATP and Reduced Pyridine Nucleotide

In contrast with aerobic metabolism where as much as 50 per cent of the substrate may be assimilated in fermenting cultures the bulk of the substrate utilized accumulates in the medium as end products of fermentation In the fermentation of a carbon compound even in growing cells as little as 5 per cent of the carbon may be utilized for the formation of cell constituents with the remainder being broken down (dissimulated) to provide energy This energy is in two main forms one is ATP the other is reduced pyridine nucleotide Several examples will serve to illustrate the manner in which these sources of energy may be utilized for the synthesis of cellular components

The hydrolysis of protein to its component amino acids is primarily an exergonic reaction and may be represented as follows

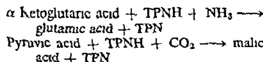

Protein + H O ⟶ amino acids + energy

On the other hand for the synthesis of proteins it is necessary that the amino acid be activated

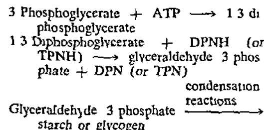

Amino acid + ATP + enzyme ⟶ enzyme AMP amino acid + pyrophosphate
Enzyme AMP amino acid + RNA ⟶ enzyme + amino acyl S RNA + AMP
Amino acyl S RNA ⟶ protein + S RNA

There is some indication that the last step the conversion of amino acyl S RNA to protein may also require energy in the form of ATP or GTP

The following examples illustrate how reduced pyridine nucleotide may be used to drive synthetic reactions which would otherwise be exergonic We have considered already the cases of amino nitrogen formation and CO_2 fixation to yield dicarboxylic acids


α Ketoglutaric acid + TPNH + NH_3 ⟶ glutamic acid + TPN
Pyruvic acid + TPNH + CO_2 ⟶ malic acid + TPN

Another example is the synthesis of glucose from 3-carbon precursors which involves both pyridine nucleotides and ATP The essential energy requiring steps may be represented as follows


3 Phosphoglycerate + ATP ⟶ 1 3 diphosphoglycerate
1 3 Diphosphoglycerate + DPNH (or TPNH) ⟶ glyceraldehyde 3 phosphate + DPN (or TPN)
Glyceraldehyde 3 phosphate ⟶ (condensation reactions) starch or glycogen

This requirement for energy is illustrated best in photosynthesizing organisms in which energy produced by light is utilized for the formation of both TPNH and ATP Both

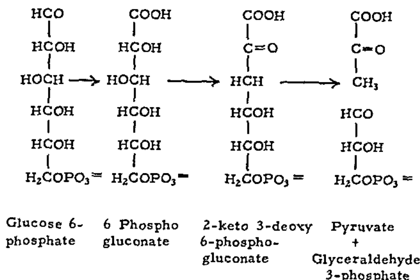
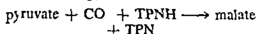
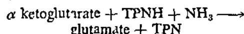


FIG 11 Glucose metabolism by the Entner Duodoroff Pathway Pyruvate and triose phosphate are further metabolized by the Embden Meyerhof pathway and the citric acid cycle

it is unlikely that it operates as a mechanism for the production of this form of cellular energy However the pathway is important for the production of another form of energy useful to the cells reduced triphosphopyridine nucleotide (TPNH) which is specifically required for a number of important synthetic processes For example it is the coenzyme utilized in the formation of C_4 acids from pyruvate and CO


$$\text{pyruvate} + CO + \text{TPNH} \longrightarrow \text{malate} + \text{TPN}$$

It also plays an important role in the formation of amino nitrogen since it can serve in the reaction catalyzed by glutamic dehydrogenase


$$\alpha\ \text{ketoglutarate} + \text{TPNH} + NH_3 \longrightarrow \text{glutamate} + \text{TPN}$$

In addition to these biosynthetic processes TPNH is known to be required for the synthesis of other reduced compounds such as fatty acids and sterols

In addition to the formation of TPNH the oxidative pathway also functions in the production of pentose for the synthesis of nucleic acids and nucleotides This can arise either by the oxidation reactions by way of 6 phosphogluconate or directly from fructose 6 phosphate by a reversal of the reactions in the left hand part of the scheme (Fig 10) The available evidence suggests that in most microorganisms pentose phosphate is formed by both mechanisms Therefore the cycle is not operating exclusively in the clockwise direction but rather in the 2 branches of the cycle the flow is in the direction from hexose monophosphate to pentose phosphate

The Entner Doudoroff Pathway This is another important alternate pathway in microorganisms (Fig 11) The first oxidative step is the same as in the pentose phosphate pathway but 6 phosphogluconate is not further oxidized to pentose phosphate but instead is split to form pyruvate and glyceraldehyde 3 phosphate The pathway was first discovered in pseudomonas It has also been detected in the *Enterobacteriaceae* and probably will be found to occur as well in other species It also occurs as a fermentation mechanism

THE DISTRIBUTION OF THE CITRIC ACID CYCLE

The presence of the citric acid cycle in bacteria has been the subject of considerable controversy and definite conclusions may be drawn with respect to only a few microorganisms It appears to function in the *Enterobacteriaceae* along with the Embden Meyerhof pathway but is absent is aerobacter and related species It probably does not occur except in greatly modified forms in most anaerobes Work in this area has been hampered by the fact that many cells are impermeable to intermediates of the

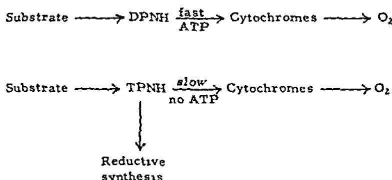


Fig 13 Oxidation of the pyridine nucleotides and production of energy

and fermentation while at the same time it was involved in the oxidation steps of the citric acid cycle and therefore of respiration On the other hand the most important steps requiring TPN were those of the pentose phosphate pathway already mentioned and isocitric dehydrogenase It has become increasingly clear that these reactions are important in generating reduced TPN for essential reduction mechanisms These reduction mechanisms were not required in the primitive atmosphere which itself was reducing and when the seas were filled with reduced cell precursors Early organisms which depended on fermentation must have possessed only a single coenzyme DPN which functioned in fermentation pathways such as the Embden Meyerhof pathway With the appearance of oxygen in the atmosphere and the consequent depletion of reducing substances of the hydrosphere the need arose to provide mechanisms for the formation of reduced cytoplasmic components and also for a source of energy less wasteful of the dwindling supply of organic materials This led to the appearance of respiratory mechanisms catalyzing the oxidation of DPNH by molecular oxygen A new coenzyme was needed to preserve the reducing potential now required by the cell This need was filled by TPNH which is not in catalytic equilibrium with oxygen In the intact metabolizing cell most of the DPN is present in the oxidized form while TPN is present largely in the reduced form Thus a cell can exist in equilibrium with oxygen and therefore at a very high oxidation reduction potential and yet maintain some of its components (such as *TPNH*) at a very low oxidation reduction state According to this hypothesis anaerobic organisms have no need for the TPN reduction mechanism and indeed it is absent in clostridia

Examples of Fermentation Pathways

Alcoholic fermentation is common in yeasts where it has been shown to proceed by a modification of the Embden Meyerhof

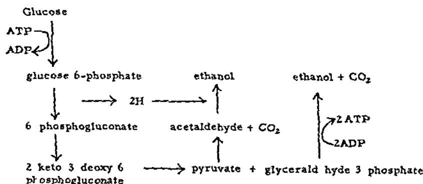


Fig 14 Ethanol fermentation of *Pseudomonas lindneri*

forms of energy are expended in accomplishing the endergonic conversion of phosphoglyceric acid to glyceraldehyde 3 phosphate (Fig 12) Fructose 6 phosphate is partially (1/6) converted to starch The remainder (5/6) is needed to regenerate the CO acceptor ribulose 1,5 diphosphate This mechanism operates in photosynthetic and chemosynthetic bacteria as well as in higher plants

Therefore in considering fermentation *mechanisms it is important to bear in mind* the requirement of the organism for these two distinct sources of energy It is also necessary to maintain balanced oxidation reductions Lavoisier first introduced the concept of fermentation when he proposed that substances rich in oxygen might undergo internal oxidations in which one part of the molecule would emerge with less oxygen and another part would become oxygen rich Thus, *Lavoisier noted that* sugar which contains 53 per cent oxygen could be fermented to a mixture of ethanol containing 35 per cent oxygen, and carbon dioxide containing 70 per cent oxygen Therefore fermentation was nothing more than an internal oxido reduction Lavoisier also proposed that all of the atoms of carbon hydrogen and oxygen must be accounted for thus stating the law of conservation of mass *The balancing of fermentation equations is* accomplished most conveniently by equating the number of hydrogen atoms produced at various stages of the process to the number consumed

Evolutionary Relation of Fermentation and Respiration Pathways

It is now generally accepted that the early atmosphere in which life originated was highly reducing in nature and devoid of oxygen and that early forms of life obtained their energy through fermentation *mechanisms Thus we may assume that* fermentation was an early evolutionary development and that respiration appeared only after the development of plant life and the resultant appearance of oxygen in the atmosphere It is instructive to consider the physiologic role of the 2 pyridine nucleotides DPN and TPN in the light of this concept DPN (cozymase) was first recognized as the coenzyme required for alcoholic fermentation TPN on the other hand was discovered in connection with the oxidation of glucose 6 phosphate to 6 phosphogluconate and first was *believed to be the coenzyme of* respiration However, with the development of our understanding of the respiratory mechanism it became clear that the substrate for respiration was reduced DPN rather than reduced TPN The oxidation of DPNH in mitochondria was found to be a rapid reaction coupled to the esterification of ATP On the other hand the oxidation of TPNH was relatively sluggish and proceeded without coupled phosphorylation (Fig 13) These discoveries led to a re evaluation of the role of the 2 pyridine nucleotides There remained little doubt about the role of DPN as the specific coenzyme of glycolysis

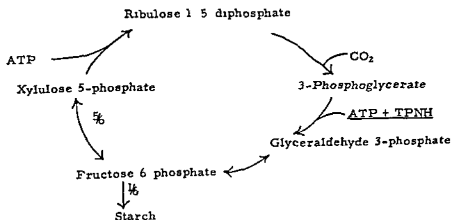


Fig 12 Energy requirement for photosynthesis Note that ATP and TPNH are employed to drive the irreversible steps *so that the cycle proceeds in the* clockwise direction

in higher organisms and the Embden Meyerhof pathway has been shown to be im portant in these organisms

Heterolactic fermentations are those pro ducing other products in addition to lactic acid They are often called mixed acid fermentations The additional products in clude ethanol acetic acid formic acid suc cinic acid carbon dioxide hydrogen butyric acid and others The metabolic origin of these fermentation products is outlined in Figure 15 In general in these fermenta tions glucose is converted to pyruvate by the usual steps of the Embden Meyerhof path way A large variety of products can be formed from pyruvate Acetoin a common product of fermentation is formed from 2 moles of pyruvate in a condensation catalyzed by the carboxylase type enzymes In some instances this is reduced to yield 2 3 butane diol Pyruvate can also be con verted by carboxylation to oxalacetate which is the precursor of succinic acid and propi onic acid In the coli aerogenes group a major pathway of pyruvate degradation in volves its cleavage to acetyl CoA and formic acid which is a common fermentation product in these organisms In the clostridia pyruvate is broken down by a different re action which produces acetyl CoA hydrogen and carbon dioxide

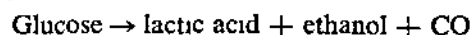
In each fermentation the balance of hy drogen equivalents must be maintained in cluding the 4 equivalents produced in the conversion of glucose to pyruvate This is illustrated best by the fermentation of hexose and pentose in lactobacilli Hexose fermenta

TABLE 1 PRODUCTS OF GLUCOSE FERMENTATION WITH *Bacillus subtilis*

PRODUCTS	MMOLES/100 MMOLES OF GLUCOSE
2 3 Butanediol	55
Acetoin	1+
Glycerol	57
Ethanol	8
Formic acid	1+
Acetic acid	tr
Lactic acid	18
Succinic acid	1+
Carbon-dioxide	118
Hydrogen	tr

tion may yield equivalent quantities of lactic acid ethanol and CO but in the fermenta tion of pentose no CO is formed and acetic acid is produced instead of ethanol In the fermentation of glucose by *Bacillus subtilis* the products formed are in approxi mately the proportions shown in Table 1 For each glucose molecule fermented we can account for 5 8 atoms of carbon 13 1 of hydrogen and 5 8 of oxygen The missing carbon and oxygen atoms are present in small quantities of acetoin formic acid and succinic acid

Heterolactic Fermentation Lactobacillus Type In many species of lactobacilli the fermentation balance is represented by the equation


Glucose → lactic acid + ethanol + CO

In *Leuconostoc mesenteroides* an organism closely related to the lactobacilli this re

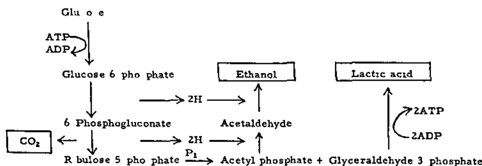


FIG 16 Hexose fermentation in *Leuconostoc mesenteroides* The fermentation products are outlined in rectangles

pathway (Fig 7) Pyruvate formed in the last step is decarboxylated to acetaldehyde and CO Then acetaldehyde is reduced to lactate The hydrogen atoms (or electrons) produced as DPNH in the oxidation of 3 phosphoglyceraldehyde are utilized for this reduction

Pure alcoholic fermentation is rare in bacteria The organism *Pseudomonas lindneri* appears to carry out a classic Embden Meyerhof type of ethanolic fermentation



$$C_6H_{12}O_6 \longrightarrow 2\,C_2H_5OH + 2\,CO_2$$

but in fact this organism utilizes the Entner Doudoroff pathway (Fig 14) The hydrogen balance is preserved since 2 atoms of hydrogen formed in the oxidation of glucose 6 phosphate are required for the reduction of one equivalent of acetaldehyde A similar balance exists in the oxidation of glyceraldehyde 3 phosphate and the reduction of the second molecule of ethanol ATP is produced in low yield two equivalents are produced in the conversion of glyceraldehyde 3 phosphate to ethanol and one of these is required for the initial phosphorylation of glucose

Homolactic Fermentation Fermentation mechanisms which give rise to lactic acid as the sole product are common in microorganisms They occur in pneumococcus as well as in many species of lactobacilli and streptococci In general the pathways may be assumed to be identical with those found

Glucose
Lactic acid ← 2H — Pyruvic acid — $-CO_2$ → Acetaldehyde — 2H → Ethanol
Pyruvic acid — CO_2 → Oxalacetic acid — 2H → Malic acid → Succinic acid — CO_2 → Propionic acid
Pyruvic acid — $-CO_2$ → Acetolactic acid — $-CO_2$ → Acetoin — 2H → 2 3 Butanediol
CO_2 2H
Clostridia: H_2 + CO_2 + Acetyl CoA
Coli-Aerogenes: Acetyl CoA + Formic acid
Acetyl CoA → Acetic acid
Acetyl CoA — 4H → Ethanol
Acetyl CoA → Acetoacetic acid — 4H → Butyric acid — 2H → Butanol
Acetoacetic acid — CO_2 → Acetone — 2H → Isopropanol

FIG 15 Origin of products in mixed acid fermentations Final products are indicated in the rectangles (Wood W A *in* Gunsalus I C and Stanier R Y (eds) The Bacteria Vol II p 82 New York Academic Press 1961)

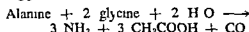
tion reduction reactions appear only at the last steps when two equivalents of pyruvate are converted to one of butyrate and 2 of carbon dioxide. The formation of butyrate by this series of reactions is a common fermentation reaction in clostridia.

In the fermentation of cysteine hydrogen sulfide is a product. Fermentations of this amino acid yield ammonia, carbon dioxide, acetate, formate, hydrogen and butyrate in addition to hydrogen sulfide.

Tryptophan is converted in part to indole and skatole, characteristic products of fermentation of mixtures of amino acids containing this substance.

Fermentation of Pairs of Amino Acids

Many species of clostridia are unable to grow on single amino acids but grow well on protein hydrolysates or amino acid mixtures. These organisms derive energy from coupled oxidation reduction reactions between pairs of amino acids, a type of fermentation known as the Stickland reaction. A typical example is the fermentation of a mixture of alanine and glycine by *Clostridium sporogenes*:



$$\text{Alanine} + 2\ \text{glycine} + 2\ H_2O \longrightarrow 3\ NH_3 + 3\ CH_3COOH + CO_2$$

In this fermentation carbon dioxide is derived from the alanine residue by an oxidative process while glycine is reduced directly to acetic acid and ammonia. A number of amino acids serve specifically as reductants in the Stickland reaction while others seem to act specifically as oxidants (Table 2). Characteristic of the Stickland

Table 2 Redundants and Oxidants in the Stickland Reaction

Reductants	Oxidants
Alanine	Glycine
Leucine	Proline
Isoleucine	Hydroxyproline
Valine	Ornithine
Histidine	Arginine
Phenylalanine	Tryptophan

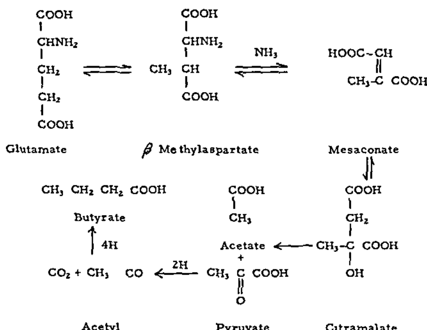


Fig 18 Pathway of glutamate fermentation (H. A. Barker *in* Gunsalus, I. C. and Stanier, R. Y. (eds.) The Bacteria, Vol. II, p. 167, New York: Academic Press, 1961)

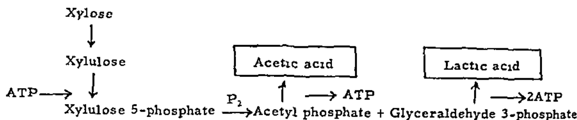
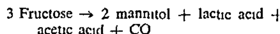


FIG 17 Pentose fermentation in *Lactobacillus plantarum*

sults from a series of reactions which constitute a modification of the hexose monophosphate shunt or pentose phosphate pathway (Fig 16) The 2 pairs of protons produced in the oxidation of glucose 6 phosphate and 6 phosphogluconate are utilized for the 2 step reduction of acetyl phosphate to ethanol In other species of lactobacilli fermentation proceeds according to the following balance



3 Fructose → 2 mannitol + lactic acid + acetic acid + CO

Since acetic acid and CO are the end products rather than ethanol and CO_2 2 pairs of protons must be disposed of in some other process This is provided by the reduction of 2 additional moles of fructose to mannitol thereby maintaining the over all fermentation balance

In the fermentation of pentoses by the same organisms the over all fermentation can be expressed as



Pentose → acetic acid + lactic acid

The fermentation mechanism resembles that shown in leuconostoc (Fig 17) Pentose is cleaved to form acetyl phosphate and triosphosphate which are the precursors of acetic acid and lactic acid respectively This type of fermentation yields 2 net equivalents of ATP per mole of pentose, the same as in the homolactic fermentation of glucose by this organism

FERMENTATION OF NITROGEN CONTAINING COMPOUNDS

Bacteria can grow on a variety of nitrogen containing compounds particularly amino acids purines and pyrimidines These provide not only energy but also carbon and nitrogen The growth of bacteria on nitrogen containing compounds generally has been associated with the production of volatile products which produce characteristic odors this type of fermentation was called putrefaction In general particularly among aerobic microorganisms amino acids are converted to intermediates of the Embden Meyerhof pathway or citric acid cycle and fermented and oxidized in the usual way Thus alanine and glutamic acid are oxidatively decarboxylated to yield pyruvic acid and α ketoglutaric acid respectively The fermentation of amino acids may involve *novel pathways which are of interest because* of the pathogenic nature of some of these microorganisms and because of the information which has been gained from the study of these pathways Thus the first evidence for the role of vitamin B_1 in intermediary metabolism was obtained through a study of the bacterial fermentation of glutamic acid

Species of bacteria which are capable of fermenting single amino acids fall primarily into 2 groups the anaerobic spore formers (clostridia) and the anaerobic cocci (micrococcus and diplococcus) A few facultative aerobes including *Escherichia coli* are able to ferment some amino acids The pathway for the fermentation of glutamate in the anaerobic spore forming organism *Clostridium tetanomorphum* is shown in Figure 18 The major products are butyric acid acetic acid and carbon dioxide The first step the conversion of glutamate to β methyl aspartate is of particular interest because of the roles of the coenzyme form of vitamin B_1 This coenzyme differs from the vitamin B_1 in having an adenosine residue attached to the cobalt molecule in place of the cyanide group The coenzyme has also been shown to function in the formation of propionic acid from succinic acid (Fig 15)

In this fermentation of glutamate oxida

this series of reactions first led to the elucidation of the role of folic acid The organism *Clostridium cylindrosporum* is remarkable in that it can grow only on the purines xanthine uric acid or guanine It is unable to use other compounds including carbohydrates for growth The fermentation of xanthine proceeds by a series of hydrolytic reactions (Fig 19) leading eventually to formiminoglycine This reacts with the coenzyme tetrahydrofolic acid to yield glycine and formimino tetrahydrofolic acid The latter is converted to 10 formyl tetrahydrofolic acid by way of the 5 10 methenyl derivative In the conversion of 10 formyl tetrahydrofolic acid to the coenzyme tetrahydrofolic acid and formic acid ATP is produced This is the first and perhaps the only energy yielding step in the fermentation It is remarkable that *Clostridium cylindrosporum* is able to fulfill all of its requirements by this series of reactions and from glycine formic acid and ATP can produce all of the complex components of the cell The fermentation of xanthine by *Clostridium cylindrosporum* has an interesting parallel in the metabolism of histidine by mammals This amino acid is ultimately converted to formimino glutamic acid (Fig 20) The conversion of formiminoglutamic acid to glutamic acid requires the conezyme tetrahydrofolic acid and yields the same intermediate formed in the bacterial fermentation In folic acid deficiency in man this reaction is blocked and large quantities of formiminoglutamic acid may be excreted This provides a sensitive and rapid method for detecting folic acid deficiency

Enrichment Culture

As may be evident from the foregoing discussion of fermentation reactions bacteria as a group are capable of utilizing any organic compound which is found in nature This has led to the development of the enrichment culture technic for the isolation of new organisms In this procedure organisms are selected which are capable of growing on the compound provided as the sole carbon source The enrichment culture technic has been used for studies of pathways of breakdown of metabolites and also to develop specific hydrolytic enzymes for the degradation of macromolecules In general bacteria will not use complex macromolecules but will first break these down to their monomeric components For example proteins will be utilized only after hydrolysis to amino

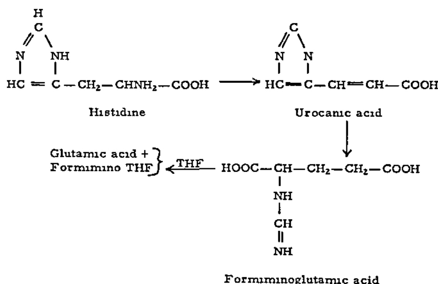


FIG 20 Role of tetrahydrofolic acid in the breakdown of histidine

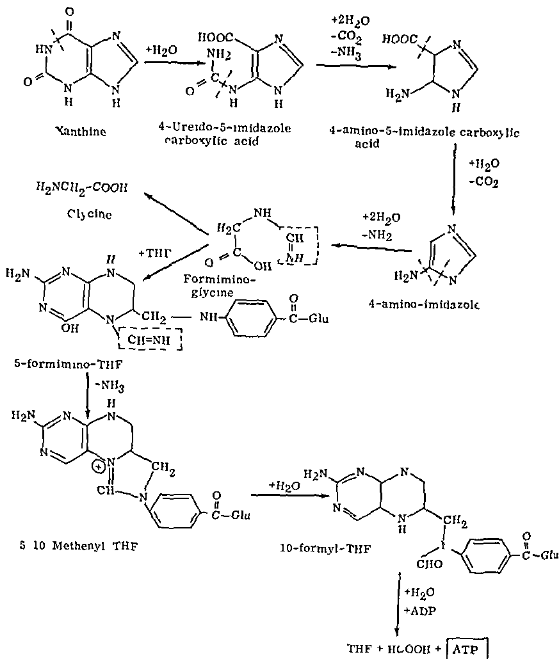


FIG 19 Fermentation of purines in *Cl cylindrosporum* THF represents tetrahydrofolic acid

reaction is the fact that the oxidants are converted directly to reduced products e g glycine to acetate proline to δ amino valerate etc For the most part the reactions leading directly to the formation of energy (ATP) in the Stickland reaction remain to be elucidated

FERMENTATION OF HETEROCYCLIC COMPOUNDS

The fermentation of purines by clostridia is of interest because it illustrates the com plex mechanisms by which organisms may derive energy from fermentation and because

may be detected by its odor or by the deposition of iron sulfide in suitable media

SYNTHESIS OF MACROMOLECULES

Bacterial cells contain nucleic acid protein carbohydrate and lipid all of which must be synthesized in more or less definite proportion in order to maintain normal cell growth

The Nucleic Acids

The synthesis of DNA is the result of the action of the enzyme DNA polymerase which catalyzes the formation of chains of deoxyribose nucleotides from nucleoside triophosphates This synthesis does not occur in a random fashion but is directed by the DNA present in the cell in such a manner that the new DNA synthesized is a precise copy of that which is originally present A schematic representation of this synthesis is shown in Figure 21 The enzyme responsible for this reaction DNA polymerase has been well characterized but the manner in which it acts inside the cell is little understood Double stranded DNA such as is normally isolated from living cells is a very poor primer and must be altered by heating or by enzyme action in order to serve effectively as a primer Once the two strands of DNA are separated each appears to act independently catalyzing the formation of a complementary strand Thus the presence of adenylic acid in the priming strand determines that thymidylic acid is inserted in the newly synthesized strand and vice versa Similarly guanine in DNA primes for the incorporation of cytosine As a consequence the over all composition of the newly synthesized DNA is an exact duplicate of that which is provided as primer

DNA synthesis appears to be the site of action of several antibiotics For example the antibiotic mitomycin inhibits completely the synthesis of DNA in intact cells while

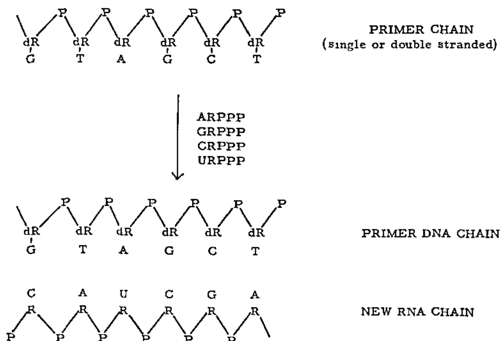


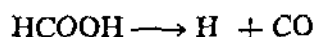
Fig 22 Schematic representation of DNA—primed RNA synthesis The symbols R and ARPPP etc represent ribose and ribose nucleoside triphosphates As in the case of DNA synthesis inorganic pyrophosphate is the other product

acids nucleic acids only after breakdown to nucleosides or purines and pyrimidines *polysaccharides* only after hydrolysis to the component monosaccharides The *hydrolytic* enzymes which microorganisms produce for this purpose have provided useful tools for the study of the structure of macromolecules

USE OF FERMENTATION PATTERNS IN THE CLASSIFICATION OF MICROORGANISMS

The classification of bacteria depends not only on morphologic examination but also to a considerable extent on the biochemical capabilities and behavior For example closely related species may be distinguished from each other by their ability to ferment specific sugars such as mannose galactose or lactose Specific media have been devel oped for these purposes when the substrate is a carbohydrate the method may depend on the production of acid through fermenta tion In aerobic organisms it is convenient to measure growth of the organism Among the staphylococci, the pathogenic species commonly will ferment mannitol while non pathogens are usually lacking in this capacity In the case of the enteric organisms shigella and salmonella species are commonly lactose negative whereas the closely related *Escherichia coli* is lactose positive As mentioned previously *Escherichia coli* can be distin guished from *Aerobacter aerogenes* by the fact the the latter is able to ferment citrate

Bacterial classification is also aided by examination of the types of products pro duced The production of gas may be due to the presence of the enzyme formic hy drogen lyase which catalyzes the formation of hydrogen and CO from formate



$$HCOOH \longrightarrow H + CO$$

The formation of acetoin is the basis for the *Voges Proskauer* reaction which helps to distinguish *Aerobacter aerogenes* Among the *Enterobacteriaceae* only *Escherichia coli* is capable of producing indole from trypto phan Another product which permits the characterization of many organisms is their capacity to form H S from cysteine This

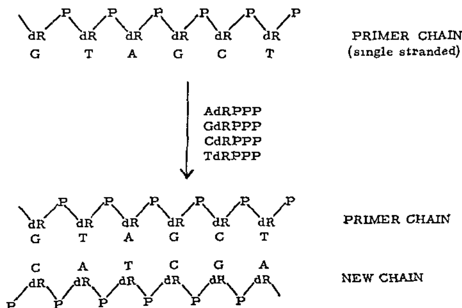


FIG 21 Schematic representation of DNA synthesis The symbols dR and AdRPPP etc represent deoxyribose and the deoxyribose nucleoside triphos phates The dotted lines connecting the bases represent hydrogen bonds Pyrophosphate is produced in the reaction

Closely related species of microorganisms differ in the location of methyl groups in the RNA and the DNA molecules

PROTEIN SYNTHESIS

The mechanism of protein synthesis in *microorganisms is considerably* better under stood than this mechanism in animal cells and may be represented by the scheme shown in Figure 23 The first step is the activation of the amino acid in the reaction with ATP to form the intermediate adenylyl amino acid This does not exist in free form but as an enzyme bound intermediate from which is transferred to a specific transfer or S RNA Specific S RNA s and specific activating enzymes exist for each amino acid thus tyrosine is converted to tyrosyl S RNA by one enzyme system and leucine to the corresponding leucyl S RNA by a distinct enzyme system and so on for each of the 18 amino acids In the reaction catalyzed by the amino acid activating enzymes inorganic pyrophosphate is produced and as in the case of DNA and RNA synthesis it is the hydrolysis of inorganic pyrophosphate that provides the driving force for what other wise would be a highly reversible process Loaded S RNA carries the amino acid to the ribosome where it is incorporated into the protein chain

Proteins which are synthesized at the ribosome possess amino acid sequences which are dictated by the information stored in the bacterial chromosome The transfer of this information from the chromosome to the ribosome involves the participation of mes senger RNA which receives the genetic code *from the DNA and transmits it to the ribo* some The language used in this system con sists of 3 letter words spelled out by the bases in the nucleic acid Thus a sequence of 3 uridine residues in the nucleic acid desig nated as UUU carries the message for the incorporation of phenylalanine The se quence AAG codes for the incorporation of glutamic acid (Table 3) In vitro synthesis of RNA by the enzyme DNA RNA poly merase results in the production of 2 strands of RNA each of which is a copy of one of the 2 DNA strands in vivo however only a single strand of DNA is copied and only a single strand of messenger RNA is pro duced This messenger RNA interacts with ribosomes forming aggregates known as *polysomes* which appear to be the actual site of protein synthesis The bacterial system appears to differ from that found in animal tissues in that the messenger RNA in bac teria is extremely unstable and protein syn thesis depends on the constanct production of new messenger RNA As a result, the entire system for protein synthesis is under close regulation and control and the syn thesis of any protein can be turned on or off almost instantly as conditions require The breakdown of messenger RNA may be related to the presence of polynucleotide phosphorylase which is found in bacterial cells but has not been positively detected in mammalian cells Polynucleotide phosphory lase catalyzes the reversible phosphorolysis of RNA to nucleoside triphosphates It has been employed in the synthesis of poly *nucleotides for study of the amino acid code*

TABLE 3 TRIPLET CODE LETTERS FOR AMINO ACID INCORPORATION GOVERNED BY MESSENGER RNA

(In each case only one of the several code letters has been given)

AMINO ACID	TRIPLET	AMINO ACID	TRIPLET
Lysine	AAA	Arginine	GAA
Asparagine	CAA	Methionine	UGA
Histidine	ACC	Glycine	GAG
Glutamine	AAC	Cysteine	GUU
Glutamic acid	AAG	Isoleucine	UUA
Tyrosine	AUU	Valine	UUG
Leucine	UAU	Phenylalanine	UUU
Threonine	ACA	Serine	UCC
Proline	CCC	Alanine	CAG

From Jukes BBRC *10* 155 (1963)

Recognition of the code for protein syn thesis appears to reside in the specific S RNA s which in turn interact with the specific amino acid activating enzymes The nature of this recognition system remains obscure but the hypothesis has been put forward that there is a triplet of nucleotides in S RNA which is complementary to the code triplet in messenger RNA and that the interaction of these complementary sets of nucleotides is responsible for the attachment of S-RNA at the proper location of the ribosome

A number of antibiotics interfere with

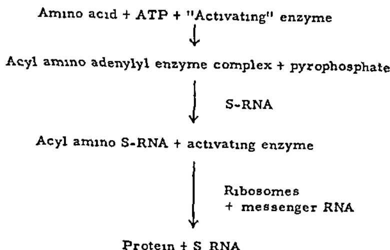


FIG 23 Pathway of amino acids in protein synthesis S RNA represents soluble" or transfer RNA The ribosomes are aggregated by messenger RNA into polysomes which are the site of protein synthesis

it leaves the synthesis of RNA and protein relatively unimpaired In cells infected with bacteriophage the effect of *mitomycin on* DNA synthesis is specific for host DNA and replication of viral DNA is not blocked This may be related to an effect of mitomycin in preventing separation of the strands of host DNA Actinomycin also interferes with DNA replication in intact cells, but this process is not so sensitive as is the synthesis of RNA

Bacteria contain several forms of RNA including messenger RNA soluble or transport RNA and ribosomal RNA Of these only the synthesis of messenger RNA is well understood This is formed in a reaction which strongly resembles the formation of DNA and is catalyzed by the enzyme DNA-RNA polymerase (Fig 22) In this reaction again DNA serves as a primer and a chain of ribonucleotides is formed with ribonucleoside triphosphates as the precursors Each base in the DNA chain directs the incorporation of the complementary base in the RNA chain adenosine in DNA governs the incorporation of uridylic acid in RNA and guanine the incorporation of cytidylic acid RNA formed in this reaction unlike the bulk of the cellular RNA possesses the same base composition as does DNA It has been established that this type of RNA turns over rapidly in bacterial cells and serves as the messenger which carries information from the gene to the site of protein synthesis (see below) Unlike DNA-polymerase RNA polymerase can be primed with either double-stranded or single *stranded DNA The activity of this enzyme* is extremely sensitive to actinomycin and it would appear that this is the primary site of action of this drug in bacterial cells

Two other types of RNA are found in bacteria These are ribosomal RNA associated with nucleoprotein particles and the so-called soluble or transport RNA The mechanism of formation of these species of RNA is not established but it has been proposed that certain regions of the chromosome specifically code for the formation of transfer and of ribosomal RNA Since these are very small regions their base composition does not reflect that of the total chromosomal DNA Transport RNA and to a lesser extent other species of RNA and DNA contain methylated bases such as thymine methyl adenine and methyl cytosine These methyl groups unlike the methyl group of thymine in DNA are derived from methionine rather than from C 1 residues of the formyl folic acid type In this respect thymine riboside differs from the corresponding deoxyriboside The methyl groups in RNA are introduced into RNA after it has been polymerized and not during synthesis of the mononucleotides The function of these unusual bases in DNA and RNA is not understood but they may be related to species specificity since the enzymes responsible for their introduction into preformed nucleic acid are highly specific

Closely related species of microorganisms differ in the location of methyl groups in the RNA and the DNA molecules

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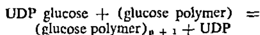
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A number of antibiotics interfere with

various steps in protein synthesis Chloramphenicol and puromycin block the transfer of amino acid from S RNA to the ribosomal protein synthesizing system Actinomycin as was pointed out previously interferes with the synthesis of messenger RNA and indirectly inhibits protein synthesis However it is noteworthy that not all protein synthesis is stopped by actinomycin Streptomycin also interferes with protein synthesis Streptomycin is known to combine with nucleic acids and it may combine with messenger RNA *thus interfering with the association* of this material with the ribosomes Streptomycin can be shown to inhibit the last step in protein synthesis in vitro furthermore ribosomes from streptomycin resistant strains of bacteria are not inhibited by the low levels of streptomycin which affect ribosomes of sensistive strains

Synthesis of Structural Polysaccharides

Bacterial cell walls and capsules contain many types of polysaccharides and the generalization can be made that the precursors of these polymers are the so called sugar nucleotides Some species of bacteria contain a substance closely resembling glycogen which probably is synthesized by the mechanism similar to that responsible for glycogen and starch synthesis in other cells



$$\text{UDP glucose} + (\text{glucose polymer}) \rightleftharpoons (\text{glucose polymer})_{n+1} + \text{UDP}$$

In this way large polysaccharides can be built up provided that the proper primers are present. Virulent strains of pneumococcus contain a variety of polysaccharides which provide the basis of group classification These complex heteropolysaccharides contain one or more of the following sugars N acetyl glucosamine galacturonic acid galactose glucose fucose and rhamnose Type III pneumococcus polysaccharide which appears to be polyglucuronic acid is built up by condensation of UDP glucuronic acid units Other precursors of pneumococcal polysaccharide are UDP N acetyl glucosamine and UDP galacturonic acid

Gram negative organisms, particularly those belonging to the *Enterobacteriaceae* also contain complex lipopolysaccharides in the cell wall This lipopolysaccharide complex *is part of the bacterial endotoxin and* O antigen and provides the basis for the Kaufmann White classification scheme for pathogenic strains Here again as in the case of pneumococcus polysaccharide the structure is built up by the successive addition of specific sugars in the form of sugar nucleotides, to the appropriate precursor The specific arrangement of sugars in the polysaccharide appears to result from the action of specific enzymes each of which *recognizes the structure required for the* addition of the next sugar The possibility remains that the antigenic portions of the molecule whose structure is genetically controlled may be coded by a kind of messenger RNA although evidence for this has not yet been provided

Lipid Synthesis

Lipid synthesis in bacteria problably follows mechanisms similar to those worked out *for mammalian cells although the details* are lacking Bacteria may contain unusual lipids for example, the lipid found in the endotoxin of gram negative microorganisms contains glucosamine rather than glycerol and a predominant fatty acid in the lipid is β hydroxymyristic acid Hydroxy fatty acids are common in bacterial systems and relatively unknown in mammalian cells Their biochemical origin has not been clarified

Biosynthesis of the Cell Wall Mucopeptide and Teichoic Acids

In the case of the muramic acid mucopeptides, which are important structural elements in the cell wall of both gram negative and gram positisve microorganisms little is known of the details of biosynthesis The basic structural unit contains β1 6 acetyl glucosaminyl acetyl muramic acid with peptide chains attached to the carboxyl group in the lactyl residue of the muramic acid component The synthesis of the cell wall mucopeptide is inhibited by penicillin and in penicillin treated cells uridine nucleotide derivatives accumulate which appear to be the precursors of the mucopeptide The steps in the synthesis of cell wall precursor are indicated in Figure 24

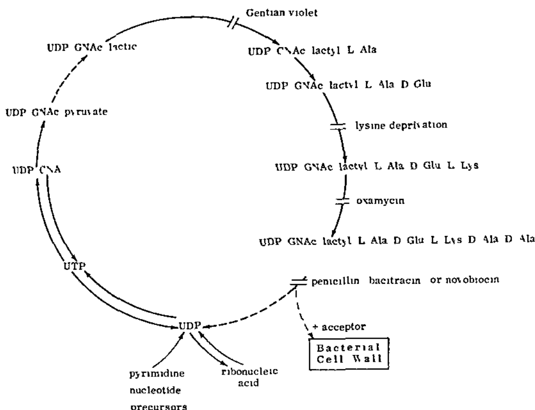


Fig 24 Cycle leading to the synthesis of bacterial cell wall mucopeptide (Strominger J L *in* Gunsalus I C and Stanier R Y (eds) The Bacteria Vol III p 445 New York Academic Press 1962)

The dye gentian violet appears to block the incorporation of the first L alanine residue into the nucleotide In cells treated with oxymycin an analogue of D alanine there appears to be a specific inhibition of the addition of the last D alanyl D alanine group which is attached as a unit rather than as single amino acids Finally the antibiotics penicillin bacitracin and novobiocin appear to block the incorporation of the completed uridine nucleotide derivative into the bacterial cell wall Unfortunately nothing is known of the mechanism of this important step in the process There is reason to believe that new cell wall is synthesized by extension of existing cell wall In lysozyme treated cells where the cell wall has been extensively or entirely removed the synthesis of new cell wall is resumed with great difficulty On the other hand if cell wall synthesis is inhibited by the presence of penicillin in which case the existing cell wall is not destroyed new cell wall synthesis begins as soon as penicillin is removed

Teichoic acid found in the cell wall of gram positive organisms is synthesized by polymerization of ribitol phosphate or glycerol phosphate the precursors are the nucleotides CDP ribitol and CDP glycerol

CONTROL OF METABOLISM

Control of Protein Synthesis

A most striking aspect of bacterial growth is that it responds rapidly to changes in the environment As has been pointed out earlier cells growing in a complex medium containing amino acids and nucleotides will grow and divide more rapidly than cells growing in minimal medium where they are required

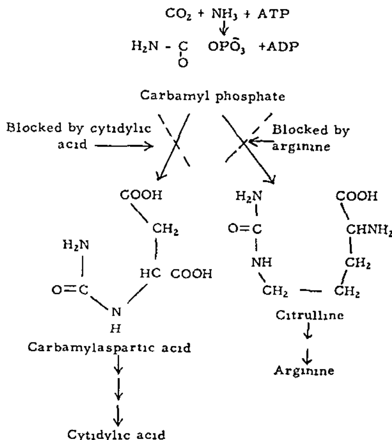
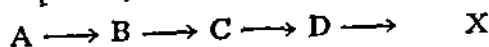


FIG 25 Specific feed back inhibition mechanisms for the control of arginine and pyrimidine synthesis The first step before the branch point is not blocked by either end product

to make all of the components needed for growth It has been demonstrated repeatedly that bacteria tend to make only those enzymes (proteins) which are necessary for growth Cells growing in a mixture rich in amino acids can be shown to lack many if not all of the enzymes required for the biosynthesis of these amino acids Therefore cells growing in a minimal medium must produce many more enzymes than cells growing in a complex medium this probably accounts for the difference in rates of growth under the two conditions

Two mechanisms are known to control protein synthesis in a specific manner One has been termed feedback inhibition and the second enzyme repression In feedback inhibition the product of a biosynthetic pathway specifically *inhibits the first enzyme in* that pathway Thus in the sequence



$$A \longrightarrow B \longrightarrow C \longrightarrow D \longrightarrow \quad X$$

X would specifically inhibit the enzyme responsible for the conversion of A to B An example of this type of feedback inhibition is found in the synthesis of cytidylic acid from aspartic acid The first step in this biosynthetic pathway is the formation of carbamyl aspartate from aspartic acid and carbamyl phosphate a reaction catalyzed by the enzyme *aspartate transcarbamylase* This enzyme is specifically inhibited by the end product cytidylic acid although this substance bears no resemblance to either of the substrates for the enzyme Characteristic of feedback inhibition is that it is generally competitive with the substrate and that it specifically blocks the first step in the biosynthetic pathway Reactions preceding the first specific reaction which may be required for other processes are not affected For example in the biosynthesis of cytidylic acid carbamyl phosphate is a precursor, but car

bamyl phosphate is also required for the biosynthesis of arginine and its synthesis is not inhibited either by arginine or by cytidylic acid (Fig 25)

Enzyme Repression

A second important mechanism for the control of biosynthesis is enzyme repression This mechanism operates at the genetic level and prevents the transfer of genetic information and therefore the appearance of specific enzymes (Fig 26) Specific repressors are formed presumably by way of specific messenger RNA although it is not yet known whether the repressor (R) is RNA or a protein formed in response to a message from RNA The repressor (R) interacts with the effector (F) which would be the end product metabolite or a product formed from the metabolite This yields the modified repressor R which is capable of interacting with the operator portion of the chromosome which controls replication of messenger RNA from the structural genes Thus in salmonella species all of the genes which govern the biosynthesis of histidine are located in one region of the cell this region is controlled by an operator located at one end of the region Interaction of the activated repressor (R′) with the operator prevents the function of the entire portion of the chromosome Since the repressor functions only if histidine is present it must be presumed that there is an interaction between the primary product of the regulator gene and histidine or a metabolite of histidine The same model can be used to account for enzyme induction or the formation of enzyme in response to a specific substrate In this case R acts as a repressor which is neutralized by interaction with the effector

Control of Nucleic Acid Biosynthesis

It is clear from the foregoing that biosynthesis of nucleic acids is itself a controlled process and that only those portions of the bacterial chromosome are active which are required for the growth of the cell under specific conditions For example organisms containing the genes for the metabolism of

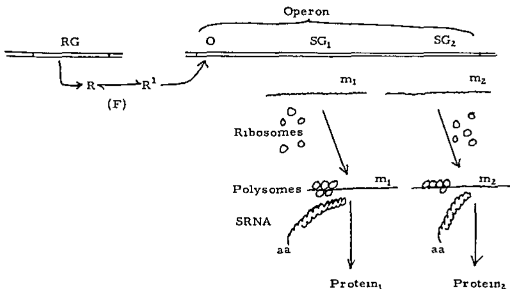


Fig 26 Model of regulation of enzyme synthesis RG regulator gene R repressor converted to R in presence of effector F (inducing or repressing metabolite) O operator SG_1 SG structural genes m_1 m_2 messenger RNAs made by SG_1 and SG aa amino acids attached to SRNA (Jacob F and Monod J On the regulation of gene activity Symposium on Quant Biol 26 193 209 1961)

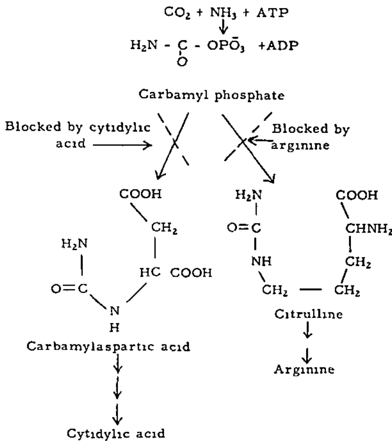
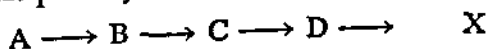


FIG 25 Specific feed back inhibition mechanisms for the control of arginine and py rimidine synthesis The first step before the branch point is not blocked by either end product

to make all of the components needed for growth It has been demonstrated repeatedly that bacteria tend to make only those en zymes (proteins) which are necessary for growth Cells growing in a mixture rich in amino acids can be shown to lack many if not all of the enzymes required for the bio synthesis of these amino acids Therefore cells growing in a minimal medium must pro duce many more enzymes than cells growing in a complex medium this probably accounts for the difference in rates of growth under the two conditions

Two mechanisms are known to control protein synthesis in a specific manner One has been termed feedback inhibition and the second enzyme repression In feedback inhibition the product of a biosynthetic path- way specifically *inhibits the first enzyme* in that pathway Thus in the sequence



$$A \longrightarrow B \longrightarrow C \longrightarrow D \longrightarrow \quad X$$

X would specifically inhibit the enzyme re sponsible for the conversion of A to B An example of this type of feedback inhibition is found in the synthesis of cytidylic acid from aspartic acid The first step in this biosynthetic pathway is the formation of carbamyl aspartate from aspartic acid and carbamyl phosphate a reaction catalyzed by the enzyme aspartate transcarbamylase This enzyme is specifically inhibited by the end product cytidylic acid although this sub stance bears no resemblance to either of the substrates for the enzyme Characteristic of feedback inhibition is that it is generally competitive with the substrate and that it specifically blocks the first step in the bio synthetic pathway Reactions preceding the first specific reaction which may be required for other processes are not affected For example in the biosynthesis of cyticylic acid carbamyl phosphate is a precursor but car

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galactose or galactosides do not possess the enzymes necessary for the utilization of the substrates unless an inducer is present in the growth medium The action of the inducer is considered to be similar to that of the repressor The control gene produces a factor controlling inducibility In the absence of the inducer this product of the repressor prevents the chromosome from expressing it self through the synthesis of the specific messenger RNA This repressor is neutralized by the inducer thus allowing the gene to function and the enzyme to be produced Enzyme induction is characteristic of cata bolic systems while repression is character istic of anabolic systems As a result of the interaction of these mechanisms with the bacterial chromosome the organism is able to cope with rapid changes in its environ ment and to respond to these changes by turning on or turning off the synthesis of specific enzymes, as the requirement of the medium may dictate

Metabolic Control Through Transport or Permeation

Bacteria are able to maintain themselves in an environment whose composition is vastly different from that found inside the cells Generally they can be considered to be quite impermeable to solutes in the medium and to possess selective mechanisms for taking up the substances which they re quire Such specific permeation mech anisms have been described for various carbohydrates amino acids and other deriva tives The permease for galactosides which has been studied extensively is specific for the disaccharide lactose and certain of its analogues It will not effect the transport into the cell of a closely related disaccharide such as maltose Conversely a specific sys tem for the transport of maltose is known which is not active with lactose or other disaccharides A variety of specific permeases have been detected for amino acids In many cases these can be shown to be inducible and to be affected by agents responsible for protein synthesis this evidence suggests that the transport mechanisms are at least in part protein in nature Furthermore they follow the classic kinetics of enzyme systems in cluding competitive inhibition substrate saturation etc Differences in the ability of organisms to utilize various external sub strates may be related to the presence or the absence of specific permeases thus *E coli* is unable to utilize exogenous citrate al though citrate is a normal intermediate in metabolism in this organism On the other hand *Aerobacter aerogenes* can utilize ex ternal citrate The difference has been shown to be due to the presence in *A aerogenes* of a citrate permease This permease is induci ble, it is not formed in cells grown on glucose The presence of specific transport or permeability systems also accounts for differing responses to antibiotics Actinomy cin will inhibit the growth of *Bacillus subtilis* since it can enter the cell and block the synthesis of messenger RNA On the other hand while the synthesis of messenger RNA in *E coli* is just as sensitive as it is in *B subtilis* in the former actinomycin is ex cluded from the cell and therefore the intact cell is resistant to this antibiotic Canavanine is an antibacterial agent in some organisms because it interferes with the utilization of aspartic acid However certain resistant mutants have lost the ability to transport canavanine which appears to be transported by the arginine permease Thus external canavanine is blocked from inter fering with the utilization of endogenous arginine Similar specific and active transport mechanisms must exist for a variety of other cell components such as salts and ions These have been little studied in bacteria

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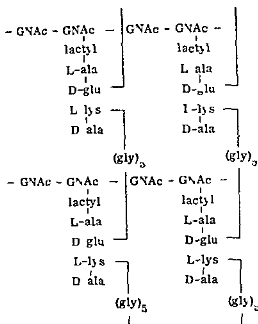


FIG 1 The cell wall of *Staph aureus* A representation of part of a macromolecular network is shown Some of the glycine cross bridges presumably go to the planes in front and back of the sheet in order to build a 3-dimensional network (Modified from Mandelstam and Strominger 1961)



FIG 2 Penicillin induced formation of *E coli* protoplasts (Hahn and Ciak 1957)

netic information proteins as catalysts and carbohydrates and fats as sources of energy Only comparatively recently has sufficient information accumulated regarding the broad similarities among cells to permit attention to be directed to dissimilarities to the chemical processes which determine that each species of organism is uniquely different from every other Antibiotics have been and will be important tools in such studies

In this chapter we shall examine the mode of action of antibacterial agents from the point of view of the types of reactions in bacteria which are inhibited and the important role which these agents have had in the uncovering and the exploration of these reactions

INHIBITORS OF CELL WALL SYNTHESIS

A remarkable chemical and morphologic difference between bacterial cells and all other types of cells is the presence in bacteria of a rigid outer layer termed the cell wall (see Salton 1960) The cytoplasm of animal plant and bacterial cells is limited by an outer membrane called the cytoplasmic membrane This membrane is relatively fragile and is the permeability barrier of the cell In this membrane are permeases which catalyze the translocation of nutrients and ions in and out of the cell In addition to this membrane bacterial and plant cells are bounded by a more rigid external layer the cell wall Some bacteria also have a third layer the capsule less defined morphologically and external to the cell wall

The cell wall of bacteria is chemically distinct from the walls of fungi and other plants It is an exceedingly complex polymer in

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6

Mechanisms of Action of Antimicrobial Agents

Without question the control of infectious diseases in the last half century albeit still incomplete is the greatest advance ever made in the conquest of human disease This advance is the consequence of the introduction of public health measures of specific immunization technics and of chemotherapeutic agents selectively toxic for bacteria but relatively nontoxic for the infected human host The possibility of finding such selectively toxic antibacterial agents had occurred to Pasteur but in 1906 the German chemist Ehrlich (see Ehrlich and Hata 1910) made the first discovery of such an agent arsphenamine the magic bullet This substance or some closely related compound was employed in the treatment of syphilis for over 30 years

The modern era of chemotherapy was ushered in by the accidental discovery of a powerful bactericidal agent penicillin by Fleming in 1929 (see Fleming 1946) and by the further success of another German chemist Domagk (1935) in discovering a synthetic chemical with a broader antibacterial spectrum than arsphenamine Prontosil * Dubos (1939) then attempted systematically to find in the soil some organism which might excrete substances toxic to other bacteria He did find two such substances tyrocidin and gramicidin which were potent antibacterial agents but which unfortunately were too toxic to be used parenterally in the human host This experience was typical Many screening programs in the past decade have led to the isolation of innumerable antibiotic producing organisms but among the substances found only a few were sufficiently selectively toxic to be used in human beings In the early 1940 s spurred partly by the need for antibacterial agents in World War II Florey and Chain undertook the isolation of penicillin (see Florey 1949) The birth of modern chemotherapy came when this purified substance was injected into experimental animals and found not only to cure infections but also to possess incredibly low toxicity for animals This fact ushered in an intense search for other antibacterial agents of low toxicity and led in rapid succession to the isolation of streptomycin chloramphenicol the tetracyclines and a whole host of other antibiotics

The thread which runs through this brief history is the two words selective toxicity They imply that the biochemical processes of bacteria are in some way different from those of animal cells and that advantage of this difference can be taken in chemotherapy All cells are of course fundamentally similar in their chemical reactions utilizing nucleic acids as the carriers and messengers of ge

* Prontosil is an azo dye and is broken down in the animal organism to p aminobenzene sulfonamide (sulfanilamide) It is the sulfonamide not the dye which is in fact the antibacterial agent

TABLE 1 EFFECTS OF PENICILLIN ON INCORPORATION OF ISOTOPES INTO CELL WALL OR INTO CELL PROTEIN AND NUCLEIC ACID IN STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI

	STAPHYLOCOCCUS AUREUS				ESCHERICHIA COLI	
	C^{14} LYSINE		P^{32} INORGANIC PHOSPHATE		H^3 DAP	C^{14} GLUCOSE
	CELL WALL	PROTEIN	CELL WALL	NUCLEIC ACID	CELL WALL	CELL WALL
Control	34 800	5 100	155 000	11 600	74 300	27 800
Plus penicillin	3 290	4 960	48 900	11 600	21 000	23 800
Inhibition per cent	91	2	68	0	72	14

NOTE Data are expressed as specific activities (c p m /mg) DAP = diaminopimelic acid (Nathenson and Strominger 1961)

inhibits wall synthesis (Table 1) The mode of killing of gram negative bacteria by penicillin appears to be fundamentally similar to the mode by which it kills more sensitive gram positive organisms Presumably a difference in the ability of penicillin to penetrate to the site of its action or differences in affinity of the binding site for penicillin account for the relative resistance of gram negative bacteria The new synthetic penicillins and the cephalosporins all appear to inhibit through the same mechanism (Rogers and Jeljaszewics 1961)

In addition to penicillin a number of other antibacterial agents inhibit cell wall synthesis A portion of the cell wall is synthesized by a reaction cycle of the uridine nucleotides (Fig 3) The inhibitors are placed at various points in this cycle with reference to the particular nucleotides which accumulate Thus penicillin bacitracin (Abraham and Newton 1958) ristocetins (Wallas and Strominger

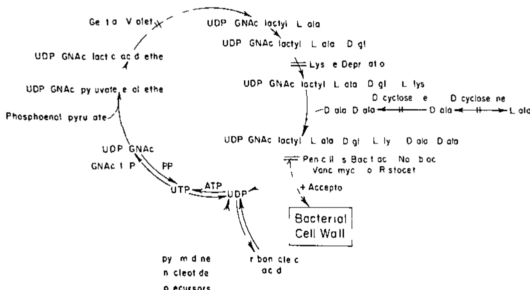


FIG 3 Reaction cycle which leads to the synthesis of part of the cell wall of *Staph aureus* Points of inhibition by various agents are indicated (Modified from Strominger 1962)

variably containing 2 amino sugars (acetyl glucosamine and a lactic acid ether of acetylglucosamine called acetylmuramic acid) and 4 amino acids (D alanine L alanine D glutamic acid and either L lysine or meso α ε-diaminopimelic acid) These are the constituents of the basal layer of the wall a polymer containing a polysaccharide and a highly cross linked peptide (Fig 1) (see Strominger 1962) * The two sugars alternate in the polysaccharide and in *S aureus* are linked by β 1 4 bonds In some species the N acetylmuramic acid residues are also O acetylated i e N O diacetylmuramic acid is present Possibly other differences between species occur in the polysaccharide However the whole structure has not yet been completely elucidated in one species and only scanty data on comparative biochemistry are available Similarly no full study of the cross linked peptide has been published but the available data indicate that there are gross differences in the nature of the cross links among species N acetylmuramic acid is a unique component of bacteria and has been found in no other type of cell Indeed its presence in *Streptomyces* has been used as a basis for classifying these organisms as true bacteria Diaminopimelic acid and the D amino acids are also unusual constitutents of bacterial cells

The cell wall is essential for the integrity of most microbial cells in their normal environment Egg white lysozyme brings about lysis by catalyzing hydrolysis of the cell wall polysaccharide resulting in solubilization of the entire wall However lysis of bacterial cells by egg white lysozyme can be prevented if treatment with the enzyme is carried out in hypertonic broths (Weibull 1953) Under these conditions the cells are converted to spherical forms lacking cell walls and called protoplasts Bacteria have unusually high internal osmotic pressures estimated to be equivalent to 5 to 20 atmospheres due to the fact that they concentrate nutrients against an osmotic gradient The hypertonic solutions are needed to counterbalance this osmotic pressure If protoplasts with their fragile limiting cytoplasmic membranes are placed in media of ordinary tonicity they explode

Penicillin can also induce this evolution of bizarre or spherical forms in bacteria (Duguid 1945 Lederberg 1956) resulting in lysis if the treatment is carried out in ordinary media However in hypertonic broth the spherical forms are stabilized (Lederberg 1956) just as are those which are produced under the action of lysozyme If the penicillin is washed out of the culture these organisms grow and revert to their normal form In one species, growth and cell division occur as a spheroplast in the presence of penicillin (Lark 1958) These observations indicate that penicillin has no deleterious effect and certainly no lethal effect on that portion of the organism internal to the cell wall and they led to the hypothesis that penicillin interferes either with the maintenance or the integrity of the cell wall

Independently of this line of investigation it was found that a uridine nucleotide containing an unusual activated sugar fragment accumulated in *S aureus* after treatment with penicillin (Park 1952 Strominger 1957) The fragment which this nucleotide contained was an acetylmuramyl peptide similar in structure to a part of the cell wall These observations suggested that the accumulated nucleotide was a biosynthetic precursor of the cell wall and therefore led also to the hypothesis that penicillin was a selective inhibitor of bacterial cell wall synthesis there by inducing nucleotide accumulation (Park and Strominger 1957 Strominger *et al* 1959)

Isotopic experiments carried out in a number of laboratories have shown that penicillin does in fact inhibit cell wall synthesis while permitting protein and nucleic acid synthesis for example to go on at normal rates thus demonstrating directly its selectivity as an inhibitor of cell wall synthesis (Gale *et al* 1958 Handock and Park 1958 Mandelstam and Rogers 1959 Nathenson and Strominger 1961) Even in *E coli* which is relatively resistant to penicillin a growth inhibitory concentration of the antibiotic selectively

* In addition to the polysaccharide and the peptide which are components of the glycopeptide all bacteria contain additional wall components referred to as special structure These components are immunologically active in higher animals as for example the teichoic acid of *S aureus* or the group carbohydrate of *S hemolyticus* or the lipo polysaccharide of *E coli*

of wall synthesis need not be its primary mode of action D-cycloserine (Ciak and Hahn, 1959 Strominger *et al* 1959) and gentian violet inhibit at earlier points and lead to the accumulation of earlier precursors

The penultimate reactions in cell wall synthesis those that lead to the utilization of the nucleotide intermediates for polymer (i e cell wall) synthesis are only beginning to be understood and only a few of the reactions which must be involved have been identified It seems unlikely that each of the five antibiotics which inhibits at this penultimate stage does so in precisely the same manner Those five compounds represent several different types of structure and among them only penicillin and bacitracin show cross resistance The most recent information suggests that ristocetins and vancomycin directly inhibit the transfer reaction involving the uridine nucleotide (Anderson and Meadow 1964 Meadow *et al* 1964) The others could interfere with other aspects of polymer synthesis e g synthesis of the acceptor (which the uridine nucleotide transglycosylase requires) with access of substrate to this acceptor with formation of the complex peptide cross bridge or even with the integrity or replication of a cell wall synthesizing particle Studies of models have suggested that penicillin may be an analogue of acetylmuramic acid (Collins and Richmond 1962) but experimental proof of this idea is still lacking

D-cycloserine inhibits at an earlier point in the cycle (Fig 3) leading to the accumulation of a uridine nucleotide which lacks two D alanine residues This antibiotic is a competitive antagonist of the utilization of D alanine for cell wall synthesis In whole cells nucleotide accumulation induced by D-cycloserine is reversed by D alanine In vitro two enzymatic reactions for which D alanine is a substrate are competitively inhibited alanine racemase and D alanyl D alanine synthetase (Strominger *et al* 1960 Neuhaus and Lynch 1964)

1 L alanine $\rightleftharpoons$ D alanine (alanine racemase)

2 2 D alanine + ATP $\rightarrow$ D alanyl D alanine + ADP + P_i (D alanyl D alanine synthetase)

The antibiotic is a structural analogue of the substrate (Fig 4) It is bound to the inhibited enzymes about 100 times as efficiently as their natural substrate D alanine Presumably the ring present in the antibiotic but absent in the substrate fixes the antibiotic in one configuration and by restricting molecular rotation in this manner either makes it relatively difficult for the molecule to come off the enzyme surface or relatively easy for it to go on the enzyme surface One of the enzymes which is inhibited alanine racemase contains pyridoxal phosphate as a coenzyme D-cycloserine is known to react with pyridoxal phosphate in a complex manner and L-cycloserine (which does not inhibit cell wall synthesis) will inhibit some reactions of L alanine involving pyridoxal phosphate enzymes The use of D-cycloserine in chemotherapy is limited by a severe central nervous system toxicity in man This toxicity could be due to a reaction with pyridoxal phosphate in nervous tissue

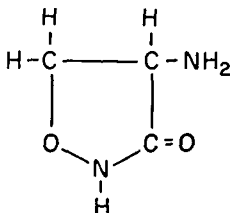
The large viruses such as meningopneumonitis and psittacosis are inhibited by both cycloserine and penicillin Cycloserine inhibition is reversed by D alanine (Moulder *et al* 1963) These viruses have cell walls containing acetylmuramic acid and they may be defective bacteria rather than true viruses

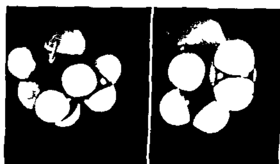
The discovery that a number of antibacterial agents are selective inhibitors of cell wall synthesis has opened a whole new area of microbial physiology to exploration These substances have provided both the conceptual background and the tools with which to obtain intermediates needed for the study of the structure and the biosynthesis of bacterial cell walls As more information is obtained perhaps it may be possible to design more effective inhibitors of cell wall synthesis or to find other ways of bringing about the death of bacterial cells as a consequence of the loss of integrity of the cell wall

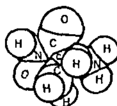
AGENTS THAT AFFECT THE INTEGRITY OR THE FUNCTION OF THE CELL MEMBRANE

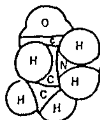
Although the cell wall is a unique component of the outer surface of cells of plants and bacteria the cytoplasm of *all* living cells is bounded by a limiting cell membrane This

1963) and vancomycin (Jordan 1961 Reynolds, 1961) all inhibit at a late stage, leading to the accumulation of the most complex of the known precursors Novobiocin also inhibits at this late stage (*Strominger and* Threnn 1959), but its effect is not so selective as that of the other substances (Brock 1962 Wishnow *et al* 1964) and inhibition

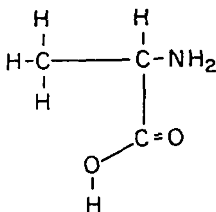


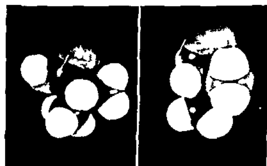


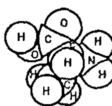


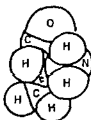


D-cycloserine









D-alanine

FIG 4 Structures (*left*) and molecular models (*right*) of D alanine and D cycloserine (Strominger J L Biosynthesis of bacterial cell walls *in* Gunsalus I C and Stanier R Y (eds) *The Bacteria vol 3 New York Acad Press pp* 413-470)

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Although the cell wall is a unique component of the outer surface of cells of plants and bacteria the cytoplasm of *all* living cells is bounded by a limiting cell membrane This

structure is the permeability barrier and has the function of controlling the internal com position of the cell Through it must pass all of the nutrients which are used to form cellu lar compounds and the ions which are con tained in the cellular environment It acts as a selective membrane to organic molecules and to inorganic ions Some organic mole cules are excluded others are concentrated by specific energy dependent mechanisms to which the name permeases has been applied Inorganic ions are also exchanged selectively so that the internal composition of the cell may be quite different from the composition of the extracellular fluid It is not surprising therefore that this important external mem brane is a site of attack for many cytotoxic agents That some of these agents are more toxic for one type of cell than for another reveals another aspect of biochemical individuality among cells and permits some of these agents to be used for chemotherapeutic purposes

The immediate onset of leakage of cellular constituents is the consequence of membrane damage and provides a simple method for measuring the effects of these antibacterial agents which have such an action Depending on the extent of damage ions, small organic molecules such as purine and pyrimidine nucleotides (which absorb at 260 mμ) amino acids and phosphate esters and even larger molecules such as protein may escape from the cell with consequent impairment of a large number of cellular metabolic activi ties The loss of these constituents is most easily followed by the loss of material ab sorbing at 260 mμ

Polypeptide Antibiotics

The cationic detergents (e g Zephiran) are the simplest examples of agents which bring about the death of bacterial cells by disrupting the function of the cell membrane A number of highly toxic polypeptide anti biotics also exert their bactericidal action through this mechanism (Fig 5) These in clude the cyclic polypeptides tyrocidin



Fig 5 Effects of polymyxin on *Pseudomonas aeruginosa* (1) Normal cells (2) Cells treated with 25 μg/ml of polymyxin This concentration causes maximum release of 260 mμ absorbing material from the cells (3) Cell treated with 500 μg/ml of poly myxin (Newton, B A J Gen Microbiol 9 54)

gramicidin polymyxins and colistin (Hotchkiss 1946 Newton 1956 Newton 1958 Chapman 1962) However some selectivity exists in their action since it is apparent that these agents can be used on superficial infections and with great caution parenterally Renal damage is a common toxic consequence but it is apparent that, although the therapeutic margin is narrow massive necrosis of animal cells does not occur at concentrations which may be bactericidal Similarly some microorganisms are not sensitive to the effects of these agents and resistant strains of previously sensitive organisms have been isolated The structure of cell membranes whether in microorganisms or in higher forms of life is virtually unknown and further information about the mechanism by which these agents disorganize the function of cell membranes must await more detailed knowledge of the structure and the function of these membranes

It may be pointed out that inhibition of cell wall synthesis in some cases may be only a special example of limited membrane damage since the cell membrane of bacteria is believed to contain the enzymes necessary for synthesis of the cell wall Although polymyxin is generally considered to disrupt membrane function in a gross manner several observations have suggested that under some circumstances it may have a more limited action on the membrane Thus *Rhodospirillum rubrum* grows as bacilli rather than in the spirillar form at low concentrations of polymyxin and at higher concentrations protoplasts are formed (Tuttle and Gest 1959) In *Neisseria catarrhalis* pretreatment with polymyxin renders the cells sensitive to lysozyme (Warren *et al* 1957) In both cases it may be inferred that polymyxin may be acting in a limited manner on one particular aspect of membrane function synthesis of the cell wall

Polyene Antifungal Antibiotics

A particularly interesting example of the selectivity of some agents for cell membrane damage is the recent demonstration that the polyene antifungal antibiotics disturb the function of the fungal cell membrane Fungi are relatively insensitive to polymyxin and tyrocidin and bacteria are insensitive to the

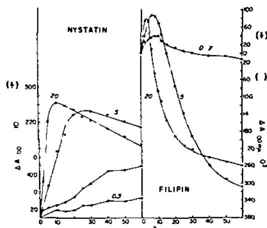


Fig 6 Shrinkage and lysis of protoplasts of *Neurospora crassa* induced by the polyene antibiotics nystatin and filipin Shrinkage (observed as an absorbancy increase) precedes lysis (absorbancy decrease) Lysis occurs more rapidly with filipin than with nystatin (Kinsky S C J Bact *83* 351)

polyene antibiotics The effect of the polyene antibiotics on permeability was observed simultaneously in two different laboratories through two different experimental approaches On the one hand studies of the metabolism of pathogenic yeasts had shown that the polyenes resulted in a severe loss of respiratory metabolism which was eventually traced to the leakage of ions and essential cofactors from the cells into the medium (Marini *et al* 1961) On the other hand studies of *Neurospora crassa* resulted in the finding that mycelial mats obtained from organisms treated with the polyenes had lost considerable weight compared with untreated controls (Kinsky 1961 1964) The weight loss was due to massive loss of intracellular constituents An elegant demonstration of the effect of polyene antibiotics is the measurement of rapid efflux of potassium from *N crassa* accompanied by diminution of the membrane potential recorded with intracellular electrodes (Slayman and Slayman 1962 Slayman 1963 Slayman and Tatum 1964)

These effects of the polyene antibiotics are due to interaction of the antibiotic with a sterol ergosterol which is present in the fungal cell membrane (Kinsky 1962a)

Bacteria do not contain sterols in their cell membranes and hence once more, an aspect of cellular individuality is seen to be the basis of selective toxicity That the lack of effect of polyenes on bacteria is due to the absence of sterols in the membrane rather than to some protective effect of the cell wall is shown by the fact that bacterial protoplasts are also insensitive to these agents By contrast protoplasts of *N crassa* are lysed by the polyenes (Kinsky 1962b) (Fig 6)

The requirement of the presence of membrane sterols for action of polyene antibiotics may also explain some aspects of the toxicity of these agents Hemolytic anemia may be a consequence of therapy with these agents The membranes of red cells contain a sterol cholesterol and red cells are lysed by low concentrations of the polyenes in a manner similar to the lysis of protoplasts of *N crassa* (Kinsky *et al* 1962) The renal toxicity of polyene antibiotics may eventually also be traced to interaction with the sterols which are present in the membranes of animal cells

The function of sterols in membranes is unknown as is an explanation of the ability of bacteria to exist without them Virtually nothing is known about the structure of cell membranes and the statement that the cyclic peptides bring about the disorganization of the lipoprotein of the membrane is simply a restatement of our ignorance of membrane structure This field is certain to be an important and exciting one in the future and the antibiotics which affect membrane function may be important probes in these studies

INHIBITORS OF PROTEIN SYNTHESIS

Chloramphenicol puromycin streptomycin tetracyclines and erythromycin are all believed to act at least in part through inhibition of protein biosynthesis The current (June 1964) concepts of protein biosynthesis outlined here are undergoing almost constant revision

The information for protein sequence is contained in DNA This information is transcribed to messenger RNA (mRNA) through a DNA-dependent RNA polymerase The machinery for transcribing this genetic message from mRNA to protein is the ribosome or an aggregate of ribosomes termed polysomes held together by a strand of mRNA The ribosomes are defined as a class of intracellular particles containing protein and RNA which in the *E coli* system at least sediment in buffered sucrose gradients containing 0 01 *M* $MgCl_2$ with a characteristic velocity and are termed 70s particles At lower Mg^{++} (10^{-4} *M*), each of these particles dissociates into a 30s and a 50s subunit and any mRNA (8-14s) which has been bound is released At still lower Mg^{++} or in the absence of Mg^{++}, further dissociation to particles smaller than 23s occurs The mRNA contains the information for protein biosynthesis in the form of a sequence of nucleotide triplets each triplet (or codon) coding for a particular amino acid Thus 300 nucleotides on an mRNA molecule would contain information for the sequence of a protein of 100 amino acids This sequence of nucleotides must be preceded and followed by periods or commas in the code which signal the beginning or the end of a new protein molecule Perhaps *each* ribosome found in the mRNA polysome complex is concerned with the synthesis of a particular protein coded in one part of the RNA molecule

The amino acids are activated for peptide bond synthesis by the formation of amino acyl sRNA ATP is required for this synthesis and is split in the reaction into AMP and PP Each sRNA molecule is specific for a given amino acid but several different specific sRNA molecules may occur for each amino acid The nonphosphorylated end of all sRNA molecules ends in adenosine and the activated amino acid is esterified to the 3 hydroxyl of the terminal adenosine In addition to components already mentioned two other protein fractions and GTP are required for protein biosynthesis

One present scheme visualizes each ribosome as containing 2 sites for attachment of aminoacyl sRNA on the 50s subunit the initial attachment at site A being determined by the codon of the mRNA molecule in position on the 30s subunit at that moment One supernatant protein factor and GTP would be required for the translocation of this aminoacyl sRNA (perhaps accompanied

by movement of the mRNA molecule along the ribosome) to a second site Then a second sRNA molecule would be bound to the ribosome at the first site and the amino acid at site B now would be transferred from $sRNA_1$ to the free amino group of amino acyl sRNA at site A with formation of dipeptidyl sRNA catalyzed by the second required supernatant protein fraction Thus by repeating this cycle the protein would be synthesized starting from its amino end (which would always remain free) Finally at some comma in the code on the mRNA the protein molecule would be released from the last sRNA molecule (by a mechanism not yet understood) with formation of a soluble protein

Chloramphenicol

In 1953 it was found that chloramphenicol inhibited the incorporation of amino acids into proteins of *S aureus* cells while permitting the synthesis of nucleic acids (Gale and Folkes 1953) In fact both RNA and DNA synthesis proceeded at normal rates (Wisseman *et al* 1954) and under some circumstances RNA synthesis was accelerated (Fig 7) Later it was found that the synthesis of cell wall and a variety of other metabolic processes were unaffected by the antibiotic This striking selectivity of action has provided a remarkable tool with which the processes of nucleic acid synthesis and protein synthesis could be dissociated and has also provided a simple means of distinguishing incorporation of amino acids into cell wall (which is insensitive to chloramphenicol) from incorporation into protein Despite the fact that this substance has been used and studied for over a decade perhaps more extensively than any other antibiotic its precise mode of action remains speculative

In an early observation of considerable importance the accumulation of RNA in the presence of the antibiotic was observed (Wisseman *et al* 1954) This so-called chloramphenicol RNA was believed by many to be an abnormal RNA since after removal of chloramphenicol from inhibited cells it was broken down before cell growth commenced again Its nature has been the subject of extensive investigation and it

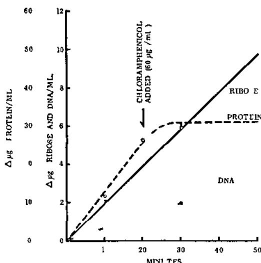


Fig 7 Effect of chloramphenicol on protein RNA and DNA synthesis in *E coli* (Wisseman *et al* J Bact *67* 662)

seems most likely at the present time that it represents a mixture of mRNA and of RNA containing ribosomal particles deficient in protein (Kurland *et al* 1962 Hahn and Wolfe 1961 Midgely and McCarthy 1962 Aronson and Spiegelman 1961) Thus 18s and 25s particles are obtained from chloramphenicol treated cells rather than the 30s and the 50s particles found in normal cells However the RNA obtained from these particles (i e ribosomal RNA) appears to be the same 16s and 23s RNA obtained from normal cells These protein poor ribosomal particles may be precursors of the normal particles and their occurrence may be a secondary reflection of a general depression of protein biosynthesis If indeed mRNA accumulates in chloramphenicol treated cells it is a remarkable fact since the instability or rapid turnover of bacterial mRNA is one of the features which distinguishes protein biosynthesis in bacteria from the same process in animals Recent experiments have suggested that the accumulated RNA is not abnormal since if the treated cells are placed in an enriched medium which permits immediate protein biosynthesis accumulated RNA does not break down (Aronson and Spiegelman 1961)

Chloramphenicol appears to dissociate



FIG 8 Molecular models of chloramphenicol (*left*) and uridylic acid (*right*) (Modified from Jardetzky 1963)

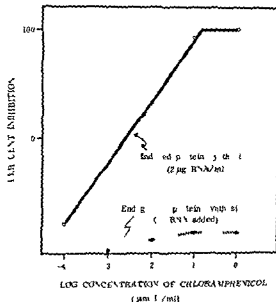


FIG 9 Chloramphenicol inhibition of ribosomal protein synthesis (rabbit reticulocyte system) induced by added mRNA Protein synthesis occurring without added mRNA and due to endogenous mRNA was not significantly inhibited by chloramphenicol In contrast the additional protein synthesis induced by added mRNA was completely inhibited by chloramphenicol (Weisberger A S *et al* *J Exp Med* *120* 161)

RNA synthesis from its normal control Normally RNA synthesis requires concomitant protein synthesis (Gros and Gros 1958 Pardee and Prestidge 1956) For example if an amino acid auxotroph is deprived of its required amino acid RNA synthesis ceases The addition of chloramphenicol under these circumstances has been observed to restore RNA synthesis and under some circumstances the rate of RNA synthesis may be greater than that which occurs normally (Aronson and Spiegelman 1962 Kurland and Maaløe 1962) An explanation of the mechanism of this effect of chloramphenicol is not now available

In cell free systems of bacterial origin chloramphenicol interferes with the incorporation of amino acids into protein However it does not interfere with the activation process i e with the formation of amino acyl sRNA and its locus of action lies somewhere in the complex reaction sequence from amino acyl sRNA to protein on the ribosomal particle (Nathans and Lipmann 1961 Rendi and Ochoa 1962) The best current hypothesis is that this substance prevents attachment of mRNA to ribosomes It has been suggested from nuclear magnetic resonance studies that chloramphenicol is an analogue of uridylic acid (Fig 8) (Jardetzky 1963) and it has been found recently that chloramphenicol inhibits attachment of poly uridylic acid to ribosomes (Jardetzky and Julian 1964) However C^{14}-chloramphenicol is bound to the 50s component of the 70s aggregate (Vasquez, 1964) while the 30s component is the site of attachment of polyuridylic acid and other mRNA molecules These facts remain to be reconciled

If the assumption that chloramphenicol is a uridylic acid analogue is correct this is indeed remarkable since its close chemical relation to phenylalanine and the fact that it inhibited protein synthesis had led earlier to the natural supposition that it was an amino acid analogue Indeed its chemical structure is extremely simple and it is the only antibiotic for which chemical synthesis is preferred to fermentation in commercial production

An explanation of the selective toxicity of chloramphenicol for bacteria as compared with animals (or fungi) is not now available It seems clear that a difference in permeability to chloramphenicol of animal and bacterial cells is not the explanation A cell free protein synthesizing system from rabbit reticulocytes is insensitive to the antibiotic (von

Ehrenstein and Lipmann 1961) and more over chloramphenicol is believed to kill bacteria which are located within animal cells

The basis of selective toxicity may lie in a difference of some detail of the bacterial and the animal protein synthesizing mechanisms The difference in stability or turnover of mRNA previously referred to is the only such difference now known Apparently chloramphenicol is far more effective in inhibiting the protein synthesis stimulated by added mRNA (e g in the form of synthetic polynucleotides) than it is in inhibiting that due to endogenous mRNA already attached to the ribosomes (Fig 9) (Weisberger *et al* 1964a Weisberger and Wolfe 1964) Therefore the difference in sensitivity of animal and bacterial systems to chloramphenicol both in vivo and in vitro could lie in a difference in stability of attachment to or turnover on ribosomes of mRNA Chloramphenicol would thus inhibit only the attachment and the function of new mRNA in bacteria but would have no influence on the function of stably attached mRNA in animal cells

Some data to support this concept have been reported Antibody synthesis must first involve the synthesis and the attachment of new mRNA and it has been observed that formation of antibody in lymph node cultures is strongly inhibited by chloramphenicol (Ambrose and Coons 1963) In treatment of infections in man with chloramphenicol one wonders whether a normal antibody response to infection would occur Similarly chloramphenicol has prolonged skin homograft survival presumably due to inhibition of new antibody synthesis (Weisberger *et al* 1964b) and one wonders whether chloramphenicol might find some use in modification of the immune response Bone marrow cells in tissue culture exhibited a marked reduction in protein synthesis after exposure to chloramphenicol for 4 to 6 days (Djordjevic and Szybalski 1960) Perhaps this time interval is required for displacement of mRNA from the ribosomes or perhaps it reflects the normal slow turnover of mammalian mRNA

Chloramphenicol is a remarkably good agent for the treatment of a number of infections and in some cases appears to be superior to other antibiotics e g in salmonella infections It is unfortunate therefore that toxicity to bone marrow cells which apparently occurs with a very low incidence but high mortality limits its general usefulness (Council on Drugs 1960) This toxicity may be related to the effects on protein synthesis described above although it is difficult at present to describe irreversible marrow aplasia in these terms However it seems likely that immature reticulocytes would be precisely the type of cell which

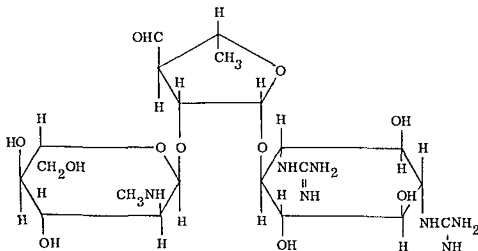


Fig 10 Streptomycin

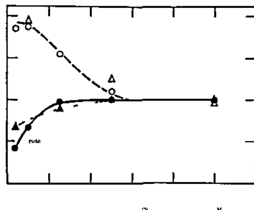


FIG 11 Effect of the streptomycin concentration in the medium on cellular composition of a dependent strain of *E coli* (Spotts C R and Stanier R Y Nature *192* 633)

would be most sensitive to chloramphenicol The maturation of reticulocytes might involve the stimulation of mRNA formation by hormonal or other means thus leading to hemoglobin synthesis as one aspect of maturation It is precisely in this kind of situation the formation of new mRNA and its attachment to ribosomes that chloramphenicol might be expected to have its greatest effect on animal cells

STREPTOMYCIN

The mode of action of streptomycin has been the subject of more controversy in the past few years than has that of any other antibiotic It has been proposed that it (1) induces a permeability defect in bacteria (Anand and Davis 1960) (2) induces synthesis of a specific streptomycin permease (Hurwitz 1964) and (3) is an inhibitor of protein synthesis (Spotts and Stanier 1961) The reader is referred to other reviews for a critical review of these various proposals The effects of streptomycin on protein synthesis will be described here because they appear to be the area of greatest current interest

Streptomycin was discovered as the consequence of a systematic search for new antibiotics soon after the importance of penicillin in chemotherapy became apparent Chemically it is composed of a base streptidine (containing 2 guanido groups) and 2 glycosidically linked sugars streptose and N methyl L glucosamine (Fig 10) Streptose is a branched deoxypentose containing a C formyl group Reduction of the formyl group yields dihydrostreptomycin which has a similar antibacterial spectrum The strongly basic character of these antibiotics is presumably important in their antibacterial activity In early work low potency of some preparations was observed and was due to the presence of an additional fermentation product mannosidostreptomycin Thus the addition of mannose to the streptomycin molecule severely reduces the antibacterial activity

Activity of this group of substances is mainly directed against some gram negative bacteria and mycobacteria but some gram positive organisms are frequently susceptible also So far no one has attempted to explain differences in susceptibility in terms of proposed mechanisms of action

Drug resistance and drug dependence occur rapidly after exposure to streptomycin and have been observed both in vitro and in vivo and any explanation of the mode of action of streptomycin must take account of the fact that resistance dependence and sensitivity appear to be multiple alleles of a single genetic locus Indeed a ribosomal locus of streptomycin action was first proposed on the basis of consideration of these facts (Spotts and Stanier 1961)

Both streptomycin and dihydrostreptomycin have been shown to inhibit protein synthesis including induced enzyme formation (Fitzgerald *et al* 1948) in intact bacteria and amino acid incorporation in reconstructed systems in vitro at extremely low concentrations (Flaks *et al* 1962 Speyer *et al* 1962) In streptomycin dependent bacteria deprivation of streptomycin leads to an increase in the amount of RNA per cell and a decrease in protein and DNA (Fig 11) This phenomenon and the accumulation of RNA in streptomycin sensitive bacteria (Dubin *et al* 1963 Dubin 1964 Stern and Cohen 1964) recall similar phenomena observed in chloramphenicol treated bacteria

As in the case of chloramphenicol streptomycin has no effect on amino acid activation but interferes with the ribosomal phase

of protein synthesis For example the polyuridylic acid stimulated incorporation of phenylalanine is strikingly inhibited (Fig 12) (Flaks *et al* 1962) Streptomycin becomes attached to ribosomes in sensitive cells Although it had been postulated that this attachment would prevent the subsequent attachment of mRNA (Spotts and Stanier 1961) it has been found that mRNA is bound even in the presence of streptomycin The resulting complex is functionally inactive (Davies 1964 Cox *et al* 1964) If mRNA (e g polyuridylic acid) is added to ribosomes before streptomycin the effect of the antibiotic is greatly reduced Incorporation due to endogenous mRNA is also not affected (Fig 12) As indicated above streptomycin is a polyanion and it may attach in the same manner or in place of Mg^{++} or polyamines Streptomycin will bind tightly to several acidic proteins (Brock 1964) as well as to polynucleotides (Cohen 1946) and either an acidic ribosomal protein or ribosomal RNA could be the site of its attachment

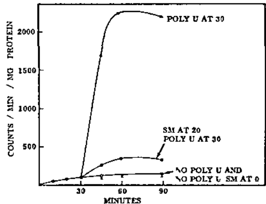


FIG 12 Effect of streptomycin (SM) on polyuridylic acid (poly U) stimulated incorporation of phenylalanine and/or incorporation due to endogenous mRNA (no poly U added) (Flaks J G *et al* Biochem Biophys Res Comm *7* 385)

The site of streptomycin resistance is the 30s ribosome (Davies 1964 Cox *et al* 1964) Ribosomes (70s) from sensitive and resistant cells were each dissociated into 30s and 50s subunits in low Mg^{++} and then 70s ribosomes were reconstituted using various combinations of 30s and 50s ribosomes from the sensitive and the resistant cells A 70s ribosome containing a 30s subunit from a resistant cell was always resistant to streptomycin while sensitivity or resistance was indifferent to the origin of the 50s subunit Thus the 70s ribosome composed of 30s (sensitive) plus 50s (resistant) subunits was sensitive to streptomycin while the ribosome composed of 30s (resistant) plus 50s (sensitive) subunits was resistant The 30s subunit of ribosomes obtained from streptomycin-dependent strains was similarly resistant to inhibition by the drug but no dependence of these ribosomes on streptomycin could be demonstrated (Cox *et al* 1964) However it seems likely that streptomycin would have become tightly attached to these ribosomes during growth in the presence of the drug

The 30s subunit is the site of attachment of mRNA It has not been directly shown that streptomycin is bound to this subunit nor has the competition for binding between streptomycin and chloramphenicol been studied However it will be recalled that the 50s subunit is the site of chloramphenicol binding

It is apparent therefore that streptomycin interferes not with the attachment but with the function of mRNA Even more remarkable is the fact that this functional impairment can be manifested as miscoding (Davies *et al* 1964) Thus in the polyuridylic acid stimulated synthesis of phenylalanine addition of streptomycin can lead to the incorporation of isoleucine Indeed variation of Mg^{++} concentration can also lead to isoleucine incorporation in the absence of streptomycin although to a lesser extent Once again these observations suggest a resemblance between streptomycin binding and Mg^{++} binding Other aminoglycoside antibiotics (kannamycin neomycins B and C) have effects similar to streptomycin but remarkably they alter the code words in different ways leading to stimulation of incorporation of other amino acids Evidence from mutants suggests that kannamycin is bound to ribosomes differently from streptomycin (Tanaka *et al* 1964) Although ribosomes from a streptomycin resistant mutant were resistant to streptomycin

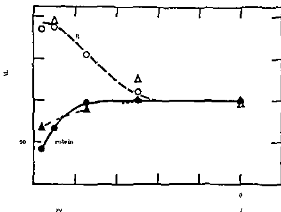


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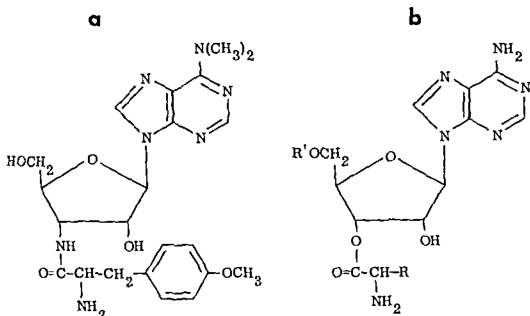


Fig 13 Structures of puromycin (a) and the adenosine end group of aminoacyl sRNA (b) R^1 = phosphodiester to the test of the RNA molecule

carried out Thus puromycin appears to be acting as a metabolic analogue of sRNA of which it is also a structural analogue replacing an incoming amino acyl sRNA molecule in the chain elongation reaction of protein or polypeptide synthesis (Fig 14) Since it is an analogue of only the end of the sRNA molecule it does not attach to the sRNA site on the 50s subunit of the ribosome Hence the polypeptide chain transferred to puromycin is released If puromycin is added during the course of protein synthesis small peptides of various sizes containing the antibiotic are released In the case of the polyuridylic acid stimulated polypeptide synthesis polyphenylalanyl sRNA is formed Since the mRNA employed (polyuridylic acid) contains no signal for the release of polypeptide from sRNA the polyphenylalanyl sRNA remains attached to the ribosome and the subsequent addition of puromycin brings about its release (Gilbert 1963) Thus in this isolated system the puromycin reaction can be used as a model of the peptide bond forming step itself It is noteworthy that this reaction is stimulated by GTP and one of the soluble protein fractions possibly the peptide bond forming enzyme (Traut and Monro 1964) Moreover this step is inhibited by chlortetracycline (see below) and chloramphenicol Whether this effect of chloramphenicol is significant or related to its other known effects remains to be investigated

It is noteworthy that puromycin does not act specifically as an analogue of tyrosinyl sRNA Differences in the actions of various synthetic analogues in which O methyl tryosine is replaced by other amino acids still remain to be explained (Nathans and Neidle 1963) Some analogues e g the glycyl analogue are relatively inactive and an aromatic amino acid appears to be important in obtaining maximum effects The transfer of the amino acid peptide linkage to the 2 or the 5 position of the ribose in the nucleoside destroys activity This structural specificity is the best evidence albeit weak that the amino acid ester is actually located on the 3 position of the ribose in the terminal nucleoside of a normal sRNA

they were sensitive to kannamycin However ribosomes from kannamycin resistant cells were resistant to both antibiotics

This phenomenon may prove to be of much use in further elucidating the ribosomal mechanism Miscoding could provide an explanation of the suppression of various mutations by streptomycin (Gorini and Kataja 1964 Lederberg *et al* 1964) Moreover suppression of a lethal mutation would be an excellent explanation of streptomycin dependence This phenomenon may also provide an explanation of the bactericidal effect of this antibiotic (Davies *et al* 1964) If the formation of abnormal and functionally inactive proteins is induced the presence of these materials could seriously interfere with the ability of the cell to survive, even in the resting state The requirement for a small amount of growth for initiation of the lethal effect of streptomycin could be explained by the fact that streptomycin can be attached to ribosomes only in the absence of mRNA Thus a single cycle of protein synthesis would release mRNA and make the ribosome accessible to the antibiotic

Finally it should be mentioned that chloramphenicol is bacteriostatic and its action is readily reversed Moreover chloramphenicol antagonizes the lethal effect of streptomycin This could be explained by a competition for binding sites or conceivably by the fact that a lethal synthesis i e the synthesis of abnormal proteins is required for killing by streptomycin, while chloramphenicol blocks this lethal synthesis in the same manner as it blocks normal protein synthesis As in the case of chloramphenicol the extreme resistance of animal cells and fungi to the action of streptomycin has not been explained adequately nor is there any explanation of the high toxicity of the amino glycoside antibiotics for the acoustic nerve of higher animals

The means of reconciliation of the observed effects of streptomycin on permeability and on protein synthesis is not immediately apparent However two possibilities suggest themselves If the ribosomal system were closely associated with the membrane both phenomena might be the consequence of binding Alternatively, if streptomycin competes for Mg^{++} or polyamine sites on the ribosome it might also compete for functionally important anionic binding sites on the membrane, perhaps Mg^{++} or Ca^{++} binding sites

Puromycin

This antibiotic is nephrotoxic in animals and is inhibitory to tissue culture cells It is not useful in the therapy of bacterial infections but related compounds have been of some interest as possible anticancer agents Although it had been observed earlier that it inhibited induced enzyme synthesis (Creaser 1955) its unusual structure first suggested that it might function as a specific inhibitor of protein synthesis It is a nucleoside in which the base is 6-dimethylamino purine (6 dimethyl adenine) and the pentose is 3 amino D ribose Moreover the amino group of the sugar is substituted in a peptide linkage with 3 O methyl L tyrosine (Fig 13) This structure suggested that this substance might act as an analogue of amino acyl sRNA (Yarmolinsky and de la Haba 1959) (Fig 13) It was found to inhibit protein synthesis in vitro It did not inhibit the formation of amino acyl sRNA but like chloramphenicol and streptomycin it blocked the ribosomal stage of protein biosynthesis (Yarmolinsky and de la Haba 1959) As the consequence small peptides accumulated in the medium (Nathans *et al* 1962) Moreover nascent protein attached to ribosomes could be released by puromycin (Morris and Schweet 1961) and with the rabbit reticulocyte ribosome system which synthesizes polypeptide chains of hemoglobin containing N terminal valine amino acid labeled puromycin was incorporated into the peptide products in an amount equivalent to the amount of N terminal valine (Allen and Zamecnik 1962) Recently, further evidence that puromycin is a part of the peptide product in the *E coli* system has also been obtained (Nathans 1964a b) Chloramphenicol acts at an earlier point in the ribosomal mechanism than does puromycin since in the presence of both antibiotics no formation of peptides characteristic of the puromycin effect can be demonstrated Analogous experiments with streptomycin have not been

RNA polymerase and DNA polymerase see different surfaces of the DNA molecule At higher concentrations of actinomycin DNA polymerase is also inhibited At these concentrations the melting curve of DNA is shifted upward (Fig 16) Apparently the double stranded configuration is stabilized and lowered activity of the enzyme is due to failure of strand separation of its DNA substrate (Reich 1964)

FIG 15 Model of actinomycin binding to deoxyguanosine of DNA in the B configuration Hydrogen bonds between actinomycin and DNA are indicated by — — and hydrogen bonds between guanine and cytosine in DNA are indicated by (Hamilton L D *et al* Nature *198* 538)

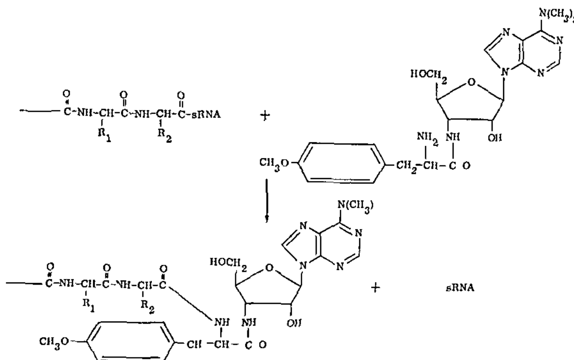


FIG 14 Proposed reaction of puromycin with ribosomal bound polypeptidyl sRNA

Other Inhibitors of Protein Biosynthesis

Tetracyclines (Gale and Folkes 1953 Rendi and Ochoa 1961 Hash *et al* 1964) erythromycin (one of a group of macrolide antibiotics) (Benigno *et al* 1954 Wolfe and Hahn 1964) streptogramin (Vazquez 1962) and lincomycin (Josten and Allen 1964) all have been reported to inhibit protein synthesis in a relatively specific manner Insufficient information is available at present to describe their mechanisms of action in detail The first two of these substances are remarkably nontoxic for animals

INHIBITORS OF NUCLEIC ACID SYNTHESIS

Actinomycin C_1

The actinomycins are bright red peptide containing antibiotics They are highly toxic for animal cells as well as for bacteria and have a useful role in therapy of some tumors They inhibit the growth of DNA viruses but not of most RNA containing viruses Their primary action is due to complex formation with DNA They do not bind to RNA The DNA actinomycin complex is strikingly inhibitory to the DNA dependent RNA polymerase but DNA polymerase is inhibited only at high concentrations of the antibiotic (Reich 1964 Goldberg and Reich 1964)

The actinomycin molecule binds to the deoxyguanosine residues in DNA Binding is readily observed as a spectral shift in the actinomycin chromophore Apurinic DNA and deoxyadenosine thymidine copolymer do not bind the antibiotic However a crab DNA which contains only 2 per cent deoxyguanosine does bind actinomycin The chromophore amino group the quinoidal ring system and the peptide chain lactone ring are all essential for the interaction An elegant model for the binding has been proposed (Hamilton *et al* 1963) (Fig 15)

DNA dependent RNA polymerase is inhibited by low concentrations of the antibiotic (Fig 16) At these concentrations DNA replication is unaffected Apparently

respiratory chain or in the phosphorylating mechanism They have permitted the isolation of portions of the respiratory chain for study and have been utilized in the preparation of fragments of the phosphorylating particles Moreover in several cases the identification of partial reactions as bona fide portions of the mechanism has been aided by their sensitivity to an antibiotic known to inhibit the intact system In addition to those already studied in detail other toxic antibiotics are known to inhibit these systems and it is to be hoped that detailed studies of their mechanisms will shed additional light on this important area of biochemistry The known differences between oxidative phosphorylation in bacteria and in animals also suggests that an antibiotic which inhibits bacterial oxidative phosphorylation and is nontoxic for animals may be found someday

METABOLITE ANALOGUES

Sulfonamides and p Aminosalicylic Acid

The modern history of studies of the mechanisms of killing of bacteria by drugs began with investigations of sulfonamides These early studies led to the discovery by Woods in 1939 1940 even before penicillin had been isolated of a naturally occurring competitive antagonist of sulfonamide action (Woods 1940) This compound was isolated and identified as p-aminobenzoic acid a previously unknown metabolite (Fig 17) Later it was found that p aminobenzoic acid was an essential part of an important coenzyme folic acid The sole metabolic function of p aminobenzoic acid is its role as a folic acid precursor Animals are unable to synthesize folic acid and depend on an exogenous source while bacteria manufacture their own folate compounds Hence only bacteria can be inhibited by sulfonamides Some bacteria do not synthesize folic acid but require it for growth These organisms like animal cells are not inhibited by the sulfonamides

More than 20 years elapsed after this discovery until more detailed knowledge of the structure and the mode of synthesis of folic acid permitted a precise definition of the inhibitory action (Brown 1962) Folate compounds are formed from pteridine precursors The immediate reaction in which p-aminobenzoic acid participates in the ATP dependent condensation of 2 amino 4 hydroxy 6 hydroxymethyl pteridine with p aminobenzoic acid to yield dihydropteroic acid which is subsequently converted to dihydrofolic acid It is this reaction which is inhibited by sulfonamides In fact sulfonamides participate as substrates so that nonfunctional analogues of dihydropteroic acid are formed These products may themselves be inhibitory and kinetics typical of pure competitive inhibition were not obtained

Another analogue of p aminobenzoic acid p-aminosalicylic acid (Fig 17) is an impor

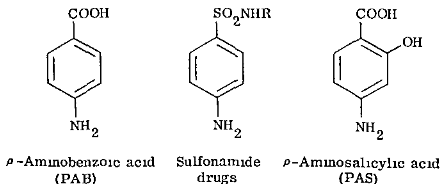


Fig 17 p Aminobenzoic acid (*left*) a sulfonamide (*middle*) and p aminosalicylic acid (*right*)

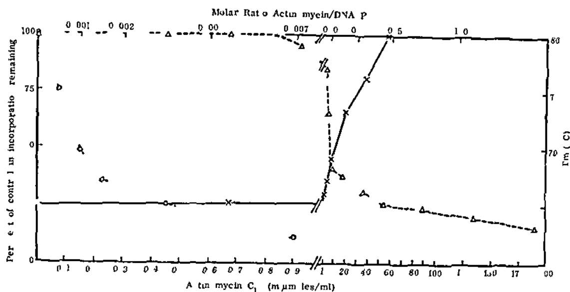


FIG 16 Effect of actinomycin on RNA polymerase (open circles) DNA polymerase (open triangles) and melting curve (T_m) of pneumococcal DNA (crosses) The incorporation of nucleotide in both polymerase reactions is plotted as a function of actinomycin concentration (lower abscissa) while the T_m is plotted as a function of the ratio of actinomycin to DNA P (upper abscissa) The latter ratios correspond to those in the enzyme reaction mixtures (Reich E *Science 143* 684)

MITOMYCIN

This antibiotic is also toxic to both animal cells and bacteria It is an effective antitumor agent and is mutagenic It brings about depolymerization of DNA The substance itself is inactive but on reduction enzymatically or chemically an active form is produced This activated substance is a bifunctional alkylating agent and brings about the cross linking of DNA strands apparently involving deoxyguanosine and deoxycytosine residues on complementary strands (Iyer and Szybalski 1963 Iyer and Szybalski 1964)

OTHER AGENTS

Phleomycin (Tanaka *et al* 1963 Falaschi and Kornberg 1964) a nitrofuran derivative (Endo *et al* 1963) phenethyl alcohol (Berrah and Konetzka 1962) and ethidium bromide (Newton 1957 Elliott 1963) are all inhibitors of DNA and/or RNA synthesis Their mechanisms of action are being studied in a number of laboratories Phleomycin like actinomycin is bound to DNA resulting in an alteration of the melting curve The binding probably involves deoxyadenosine or thymidine residues While the binding of this antibiotic inhibits DNA polymerase the DNA-dependent RNA polymerase is not affected (Falaschi and Kornberg 1964) This situation is just the opposite of that seen with actinomycin C_1

INHIBITORS OF OXIDATIVE *PHOSPHORYLATION*

A number of antibiotics highly toxic for both animals and bacteria, have been found to be inhibitors of oxidative phosphorylation Although studies of the mechanism of oxidative phosphorylation have shown that there are important differences between the bacterial and the animal systems no inhibitors of this kind have been found so far which are selectively toxic for bacteria However those agents which have been found have been extremely useful in studies of oxidative phosphorylation especially antimycin A oligomycin aurovertin and gramicidin (Hotchkiss 1944 Brodie and Gray 1956 Chance 1956 Lardy *et al* 1958 Lardy 1961) These inhibitors act at specific loci in the

peutic agents now used and of the basis of their toxic side-effects in animals Perhaps it will even lead to the design of more effective chemotherapeutic agents than those now available but without question such studies have contributed and will contribute in an important way to that fund of basic knowledge on which modern science including medicine is based

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*Two excellent and comprehensive reviews of the subject of this chapter have been published recently (Davis and Feingold 1962 Gale 1963) and interested readers are referred to them for a more detailed treatment of some aspects as well as for additional references

tant antitubercular drug It is remarkable that while *Mycobacterium tuberculosis* is effectively inhibited by p aminosalicylic acid sulfonamides have no inhibitory action Correspondingly sulfonamide sensitive bacteria are by and large insensitive to p aminosalicylic acid Like sulfonamides, p aminosalicylic acid may also be utilized by sensitive organisms in vivo at least to form folic acid analogues

These observations imply that the receptor site for p aminobenzoic acid on the sensitive enzyme may be different in different organisms in one case being fit by sulfonamides and in another by p aminosalicylic acid Indeed alterations in enzyme receptor sites for p aminobenzoic acid are one mechanism by which cells of *E coli* become sulfonamide resistant (Pato and Brown 1963) In view of the importance of p-aminosalicylic acid as an antitubercular drug and the high frequency with which resistance to it develops it is surprising that this system has barely been studied in mycobacteria It may be hoped that the description of the enzymatic mechanisms involved will stimulate further work in this area

The discovery of the p aminobenzoic acid sulfonamide antagonism led to the general concept of antimetabolites as potential therapeutic agents and to the hope that this concept would permit the rational design of new antibacterial substances Although a large number of compounds of various types have been made and tested none of them has possessed the selectivity required for this purpose *

Other Substances

A number of other antibiotics are antimetabolites Thus diazo-oxo norleucine (DON) and azaserine are glutamine analogues (Hartman *et al*, 1956) and hadacidin is an L aspartic acid analogue (Shigeura and Gordon 1962) Psicofuranine is an adenosine analogue, containing the keto sugar psicose rather than ribose This latter is of particular interest because it illustrates a novel mechanism of antimetabolite action (Moyed 1961) The antibiotic is an inhibitor of xanthosine 5 phosphate aminase which catalyzes the formation of guanylic acid XMP + ATP + $NH_3 \rightleftarrows$ GMP + AMP + PP This enzyme has an allosteric regulatory site for which AMP may be the normal regulator The antibiotic appears to inhibit the enzyme by acting at the regulator site Several synthetic amino acid analogues have a similar action in inhibiting amino acid biosynthesis

* A striking exception to this statement in another area of chemotherapy is iododeoxyuridine This compound was originally synthesized as a nucleic acid antimetabolite in a search for anticancer agents and does have limited use in the therapy of some tumors However it is a potent inhibitor of the replication of *Herpes simplex* a DNA-containing virus Infection of the eye with *Herpes* is an important cause of blindness and local instillation of iododeoxyuridine in the eye produces a rapid cure of this disease (Kauffman *et al* 1962) This compound is the first antiviral agent of value It is also being tried in the treatment of smallpox

CONCLUSION

In concluding a chapter on the mechanism of action of antimicrobial agents one pauses to reflect on the enormous effort which has been expended in this area of investigation This has been due largely to the fascination of scientists with antibiotics as keys with which to unlock new areas of microbial physiology These efforts have been richly rewarded Studies of sulfonamides led to the isolation of p aminobenzoic acid and studies of penicillin led to the full realization of the functional importance of the bacterial cell wall Chloramphenicol and other antibiotics have been exceedingly important in unravelling the mysteries of protein and nucleic acid synthesis and perhaps the polyenes and other antibiotics will play a similar role in the eventual understanding of membrane structure and function It is of interest that most of the agents studied have interfered in one way or another with the macromolecular components of bacterial cells and that simple competitive antagonism is a relatively unusual phenomenon The concept of biochemical individuality among cells is slowly being defined partly with the aid of these substances and indeed is the basis of the selective toxicity of those antibiotics which are useful in chemotherapy It is to be hoped that these fundamental investigations will lead to a better understanding of the thera

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7

Pathogenic Properties of Bacteria

INTRODUCTION

Our purpose in this chapter is to discuss examples of factors possessed by bacteria that enable them to cause disease Much has been written about commensalism and parasitism between microorganism and animal or plant host It has been stressed that disease is a rare event in the normal parasitic relationship and therefore is a minor aspect of parasitism in which peaceful coexistence is averred to be the rule On the other hand depression of the host s resistance by ionizing radiations for example shows clearly that bacteria which we are wont to consider as commensals, the normal flora of the gut under usual circumstances are prevented from invading the tissues to cause disease and death only because the protective mechanisms are intact Furthermore many of the species of microorganisms that cause disease are not found in a benign parasitic relationship as for instance the viruses of influenza measles and smallpox in man or rinderpest in cattle When these agents have been introduced into a population overt disease is the rule unless specific prophylaxis by immunization has been practiced or the population has become immune because of previous epidemic infection Between the two extremes of parasites which usually are commensal and those that usually cause widespread disease in the unimmunized there is a spectrum of relationships in which the frequency of disease depends on an unsteady balance between the state of the host and the pathogenicity of the numberless microbial species or strains that are encountered

From the standpoint of the host the aim is to preserve homeostasis and a variety of protective mechanisms are available to achieve this end Homeostasis can be maintained despite the presence of large and varied microbial populations provided that they are restricted to certain regions of the body where they can be tolerated However, if for one reason or another there is a defect in the homeostatic barriers and the microorganisms gain access to other tissues disease results The relation betwen microbe and host is dynamic whether the species of microorganism is classed as pathogen or commensal The host appears to do everything in its power to rid itself of the parasite and if this is not possible at least to restrict it to a region where it can do the smallest amount of harm or occasionally be of benefit because the parasite is uniquely able to synthesize nutritional factors necessary for the host or possesses enzymes capable of cleaving ingested materials to a state in which the host can utilize them

In summary it can be said of the relations between host and microorganism that in some instances there is a state of relatively peaceful coexistence or commensalism in others there is an uneasy truce that is commonly broken and in the case of highly pathogenic microbial species the usual state is open warfare Whatever military figure of speech is used to describe the complex relationships

it is clear that no single generalization can apply to all but that each case needs to be analyzed separately as has been done in subsequent chapters

PATHOGENIC PROPERTIES OF BACTERIA

The term *pathogenicity* refers to the capacity of microorganisms to cause disease either natural or experimental in a given host species Pneumococcus is pathogenic for the mouse although spontaneous pneumococcal disease never has been observed in this species Nonetheless the mouse is extremely susceptible to experimental infection and rapidly succumbs following injection of only a few bacteria In order for disease to occur naturally in a population pathogenic bacteria must be endowed also with certain attributes which will be discussed under the collective term *communicability* although it should be borne in mind that factors pertaining to the host or to intermediate vectors in addition to intrinsic properties of the microbes play a large part in transmission of infection from one individual to another

Pathogenicity and virulence have commonly been used as synonyms but as Miles (1955) has pointed out a useful distinction can be made between the terms Pathogenicity may be regarded as a general attribute of a species or genus or some other grouping of microorganisms the term *virulence* being used to indicate the degree of pathogenicity of a given type strain or clone as observed in a particular host species under defined conditions *Bacillus anthracis* is pathogenic for many genera of animals Mutant strains or variants of anthrax bacillus show great differences in virulence or relative pathogenicity when tested in guinea pigs for example and the differences in virulence are stable characteristics of the variants These then are virulent or avirulent strains of the pathogenic species *B anthracis* Pathogenicity of pneumococcus as a species is attributable to the presence of antiphagocytic polysaccharide capsules which surround the cells In the same animal species mouse enormous differences in virulence occur as characteristics of different capsular types Moreover within a single type stable mutants exhibit high or low virulence correlated with synthesis of large or small amounts respectively of capsular polysaccharide

Although the virulence of a specified stable strain of a microbial species can be measured with relative reproducibility in a given inbred strain of laboratory animal it is common place to find quite different results when other strains of the same animal species are similarly tested It follows that the results found in one animal species should not be extrapolated to another

Virulence can be expressed only in comparative terms because of variations in both host and parasite To arrive at even a rough numerical value for the virulence of a newly isolated microorganism be it bacterial viral or fungal requires the use of large numbers of animals infected with increments of dosage and whenever possible by comparison with the results found with a calibrated strain of the same species tested at the same time Then a numerical comparison of virulence for the animal species under test can be made based either on the number or the dose of infectious agents required to produce a pathologic effect or else by differences in the effect caused by similar doses of each microbe

The nature of the effect chosen as the indicator of virulence may vary from the number of microorganisms or colony producing units found per unit weight of a selected infected tissue to an estimate of the number and the extent of the lesions produced Death of the animal usually provides the most secure end point

The concept of a minimum lethal dose (M L D) of microorganisms as a measure of virulence may be grossly misleading because it does not take account of variations in resistance of different members of the host population of variation in virulence among the microorganisms inoculated nor of differences in response to increments of dose Death rates resulting from constant increments of dose in similar groups of a population of animals are found usually to increase slowly in the range of 1 to 10 per cent and 90 to 99 per cent mortality and most rapidly in the region of 50 per cent mortality For this reason the dose that kills

50 per cent of the animals can be estimated more precisely than at either extreme of the mortality curve As a result the median or 50 per cent lethal dose (LD_{50}) is used most commonly in the description of virulence Calculation of the LD_{50} can be made by employing a formula such as that devised by Reed and Muench (1938)

In infectious processes where death can not be used as the endpoint, other pathologic effects can be related to the dosage of microbes required to produce them in 50 per cent of the test animals in a given time This dosage is often referred to as the median or 50 per cent infectious dose (ID_{50})

Although estimation of the median lethal dose of certain well studied strains of microbes can be made in the laboratory with fair reproducibility it should be emphasized that this measurement may bear little relation to virulence of a species of bacterium in the natural state

MICROBIAL FACTORS THAT DETERMINE PATHOGENICITY

The production of disease involves interaction between microbe and host and for this reason discussion of the pathogenic properties of the infective agent requires also a consideration of factors of host resistance Recognition that bacterial products are concerned in virulence depends on the specific pathologic effects they produce as well as on the immunologic response of the host to the infective agent or fractions of it

Bail and Weil (1911) introduced the word aggressins to describe nontoxic factors produced by *B anthracis* and other bacteria that interfere with host resistance and cause the production of specific, protective antibodies The usefulness of the term is debatable The same may be said for the expression aggressive factors which have been defined as substances produced by microorganisms that assist them to establish or extend the infection by inhibiting phagocytosis by breaking down mechanical barriers to spread or by killing the host s tissues The expression aggressive factors is so comprehensive that it could almost be used as a synonym for pathogenic factors However because of the confusion that might result the terms pathogenic factors or virulence factors will be used in the present discussion

It should be understood clearly that the factors which govern the pathogenicity of one bacterial species may be entirely different from those of another species This may be illustrated by contrasting the factors concerned in the pathogenicity of *Clostridium tetani* and pneumococcus

If the spores of *Cl tetani* are introduced into a wound together with some material capable of causing local tissue damage germination takes place, and growth of the vegetative form of the bacillus occurs locally During growth a highly poisonous and freely diffusible protein tetanus toxin is elaborated by the bacteria The toxin travels centripetally along peripheral nerves probably in the interneuronal tissue spaces (see Wright 1955) reaches the central nervous system where it affects the motor neurons and produces the frequently fatal disease tetanus The bacteria themselves are present only at the original site of introduction All the characteristics of the natural disease can be reproduced in animals by injecting minute doses of highly purified tetanus toxin, immunization by means of toxoid prepared from the toxin prevents the disease *Cl tetani* possesses a single factor tetanus toxin which appears to account for its pathogenic qualities

The course of events differs greatly when a few virulent pneumococci are introduced into the tissues of a susceptible animal The microorganisms multiply rapidly invade the blood stream and are carried throughout the body At death they are found in large numbers in all organs in striking contrast with the animal infected with tetanus bacilli The capacity to invade and multiply in the tissues is determined by the polysaccharide capsule which surrounds virulent pneumococci This viscous substance inhibits ingestion of the microbes by phagocytes apparently in a mechanical way and is not toxic for the isolated phagocytes or for the intact animal even though injected in very large amounts No toxin in any way analogous to tetanus toxin is known to be produced by pneumococcus Furthermore antibodies to the purified polysaccharides confer complete protection against infection

Therefore the single demonstrable patho

TABLE 1 FACTORS THAT INFLUENCE PATHOGENICITY OF SOME BACTERIAL SPECIES

BACTERIAL SPECIES	NATURE	SITE OF ACTION	MODE OF ACTION	SPECIFIC IMMUNITY TO INFECTION	COMMENT
Pneumococcus	Capsular polysaccharide	*Phagocytes*	Inhibits phagocytosis	Antipolysaccharide antibodies	Antibodies enhance phagocytosis No significant toxin identified
Hemophilus influen ae	Capsular polysaccharide	Phagocytes	Inhibits phagocytosis	Antipolysaccharide antibodies	Role of endotoxic factors unknown
Streptococcus pyogenes	M proteins at cell surface	Phagocytes	Inhibits phagocytosis	Anti M protein antibodies	Hyaluronic acid is not antigenic Antibodies to various toxins do not prevent infection but may mitigate it, e g antierythrogenic toxin prevents rash of scarlet fever antistreptokinase inhibits digestion of fibrin by streptokinase plasminogen plasmin system
	Hyaluronic acid capsule	Phagocytes	Inhibits phagocytosis		
	Numerous exotoxins and extracellular enzymes	Multiple blood vessels red blood cells leukocytes myocardium etc	Cytotoxic hemolytic various hydrolytic enzyme reactions		
Bacillus anthracis	D polyglutamic acid capsule	Phagocytes	*Inhibits phagocytosis*		Antibodies to capsular material not protective See *text*
	Extracellular toxin	Generalized	Damages phagocytes produces edema and necrosis shock oligemia and death	Antibodies to toxin or possibly to spontaneously toxoided derivatives	

TABLE 1 FACTORS THAT INFLUENCE PATHOGENICITY OF SOME BACTERIAL SPECIES (*Continued*)

BACTERIAL SPECIES	NATURE	SITE OF ACTION	MODE OF ACTION	SPECIFIC IMMUNITY TO INFECTION	COMMENT
Pasteurella pestis	Envelope or capsular antigen (Fraction I) carbohydrate protein complex	Phagocytes	Inhibits phagocytosis		Fraction I evokes protective antibodies in rats mice rabbits and monkeys when given in saline or oil protective to guinea pigs if given in oil in doses not exceeding 500 μg antibodies to VW protect mice against infection by virulent strains that lack envelope antigen role of Fraction I in mouse virulence is not clear
	VW antigen complex	Pha ocytes	Inhibits phagocytosis of *P pestis* by mouse cells	Antibodies to envelope antigen (Fraction I) antibodies to VW complex in mice	
	Extracellular protein toxin	Phagocytes liver (?) kidney (?)	Prevents destruction of *P pestis* by damaging phagocytes		
Corynebacterium diphtheriae	Exotoxic protein	Cells of most tissues	Necrotizing inhibits protein synthesis inhibits oxidative processes possibly through interference with cytochrome b	Antitoxin	Toxin produced following infection with appropriate diphtherial bacteriophages
Clostridium botulinum	Exotoxic protein	Cholinergic nerve endings in peripheral autonomic and somatic fibers	Anticholinergic inhibits release of acetylcholine	Antitoxin	Six types of *Cl botulinum* A B C D E F produce immunologically distinct toxins
Clostridium welchii	Lecithinase exotoxin	Lecithin of cell membranes and mitochondria	Cytolytic	Antilecithinase	

	Collagenase	Collagen framework of intact tissues	Proteolytic		Destroys collagen framework in intact muscle Antibodies to collagenase and *hyaluronidase not* protective against infection
	Hyaluronidase	Intercellular ground substance	Depolymerizes hyaluronic acid		
Clostridium tetani	Exotoxic protein	Motor neurons in cerebrospinal axis	Anticholinergic (?) abolishes *synaptic* inhibition	Antitoxin	Toxin is bound by ganglioside of nervous tissue
Salmonella typhosa	O antigens polysaccharide protein phospholipid complex	Circulating phagocytes and RES cells serum factors small blood vessels	Inhibits phagocytosis inhibits complement modifies fibrinogen potentiates effects of epinephrine etc	Antibodies to cells containing *O antigen*	
	Vi antigen (polysaccharide)	Phagocytes	Antiphagocytic		

TABLE I FACTORS THAT INFLUENCE PATHOGENICITY OF SOME BACTERIAL SPECIES (*Continued*)

BACTERIAL SPECIES	NATURE	SITE OF ACTION	MODE OF ACTION	SPECIFIC IMMUNITY TO INFECTION	COMMENT
Pasteurella pestis	Envelope or capsular antigen (Fraction I) carbohydrate protein complex	Phagocytes	Inhibits phagocytosis		Fraction I evokes protective antibodies in rats mice rabbits and monkeys when given in saline or oil protective to guinea pigs if given in oil in doses not exceeding 500 μg antibodies to VW protect mice against infection by virulent strains that lack envelope antigen role of Fraction I in mouse virulence is not clear
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	Vi antigen (polysaccharide)	Phagocytes	Antiphagocytic		

genic factor in pneumococcus the capsular polysaccharide differs entirely from tetanus toxin both in its nature and mode of action and the mechanisms by which these two bacterial species cause disease are quite different Tetanus is a highly fatal disease caused by a toxic metabolic product of a microorganism *that is incapable of multiplying in healthy* tissue whereas in pneumococcal infections the invasive bacteria cause death only after extensive multiplication in tissues of the host

Many diseases caused by bacteria fall into either one of the two categories illustrated by the foregoing examples However a larger number belong to a third category in which the microorganisms exhibit multiple pathogenic qualities as shown in Table 1

Possession of an antiphagocytic component such as a capsule does not mean that this material is the most significant factor in virulence This is well illustrated by the anthrax bacillus in which all naturally occurring virulent strains are found to be encapsulated on the other hand encapsulated strains may be avirulent It is apparent that other factors have overriding importance as compared with the capsule

To determine precisely the part that a *particular cell component plays in the pathogenicity* of a bacterial species may be very difficult In pneumococcus the situation is deceptively simple since invasiveness can be shown by a variety of methods to depend on a single morphologic structure the polysaccharide capsule which is antigenic Moreover *complete protection against infection* is provided by antipolysaccharide antibodies In general demonstration that antibodies to a cell fraction are protective has afforded the most secure basis for inferring that it determines or influences pathogenicity In cases such as the anthrax bacillus in which antibodies to the polyglutamic acid capsule do not prevent *infection more indirect methods* must be used such as the demonstration in vitro that the capsule inhibits phagocytosis In Group A and Group C streptococci the hyaluronic acid capsules are not antigenic but appear to influence pathogenicity because it has been shown that infected animals may be afforded a degree of protection if they are injected with the enzyme hyaluronidase which digests away the hyaluronic acid capsule in vivo

If antibodies to a cell product prevent infection this is very strong evidence that it is a virulence factor For example, antibodies to the M proteins on the surface of Group A streptococci are protective However it is *much more difficult to specify the role of* other cell products such as toxins or extracellular enzymes of streptococci in the infectious process because antibodies to them do not prevent disease although they may modify its course Demonstration that an isolated product is toxic such as the O hemolysin of several bacterial species cannot be taken to mean that it has significance as a determinant of pathogenicity At the same time one should not infer that such products have no influence on the course of disease on the grounds that antibodies to them do not prevent *initiation* of infection It would seem to be more reasonable to consider the M proteins as the primary determinants of virulence and the other cell products as secondary candidates that may come into operation after infection has been established

Pathogenic staphylococci produce during growth both in cultures and in the course of *naturally occurring infections a relatively* potent toxic protein termed alpha toxin Nonpathogenic strains do not produce it As little as 1 mcg of this substance is capable of killing a mouse (Bernheimer and Schwartz 1963) and about the same quantity injected into the skin of a rabbit results in the local *development of an impressive necrotic* lesion Striking damage in vitro to erythrocytes leukocytes platelets and tissue-culture cells can be demonstrated Despite these properties the extent to which alpha toxin contributes to the pathogenesis of staphylococcal disease is not clearly understood for some humans who have substantial amounts *of circulating antitoxin are nevertheless* susceptible to staphylococcal disease and the degree of protection afforded by specific antitoxin to artificially infected animals seems to depend on the experimental conditions selected by the investigator

The species listed in Table 1 have been chosen to exemplify the multiplicity of factors that are involved in the *pathogenicity of*

bacteria and to indicate something of their nature and mode of action Complete information is not available for any species Even in the case of pneumococcus which has been studied more extensively than any of the other species shown in Table 1 we are entirely ignorant of the factor or factors produced by it which cause death of the infected host It is abundantly clear that the nontoxic polysaccharide capsules are of primary importance in enabling pneumococcus to be invasive and equally plain that death of the host cannot be ascribed to a direct toxic action of the polysaccharides

1 *Nontoxic antigenic surface components which act by inhibiting phagocytosis and antibodies to which are protective* The polysaccharide capsules of pneumococci already referred to are examples of this category Through inhibition of phagocytosis they permit multiplication of bacteria in the tissues Antibodies to them promote phagocytosis and protect against infection Many other important pathogens such as *Hemophilus influenzae* and *Klebsiella pneumoniae* likewise owe their chief pathogenic activity to the protection against phagocytosis afforded by antigenic polysaccharide capsules

A surface antigen of *Staphylococcus aureus* consisting principally of polysaccharide has been isolated by Morse (1962) This substance appears to be responsible for resistance to engulfment by phagocytes because absorption of antiserum with it removes opsonizing antibody moreover injection of the antigen into mice results in protection against intraperitoneal challenge with the homologous strain However the antigen appears to be absent from a number of other strains of *Staph aureus* and the general significance of the observations has still to be evaluated

Proteins on the bacterial surface may act in a similar way Group A streptococci (*Streptococcus pyogenes*) can be differentiated into many types based on immunologically distinct acid soluble M proteins which are present at the cell surface Although Group A streptococci produce many other components toxins and enzymes which may play a pathogenic role once infection has been established the ability to initiate the disease process depends in the main on the antiphagocytic activity of the M proteins Moreover anti M antibodies are protective whereas antibodies to other cell products although they may modify the disease do not prevent infection

Naturally occurring fully virulent *Pasteurella pestis* produces a surface material referred to as envelope antigen capsular antigen or Fraction 1 which inhibits phagocytosis It appears to be a carbohydrate protein complex Antibodies to it protect against experimental infection in mouse rat guinea pig monkey and presumably in man (for review see Burrows 1955) Burrows (1957) has described a second antiphagocytic material termed the VW antigen complex which is also toxic for leukocytes Mouse virulent mutants of the plague bacillus occur that lack capsular antigen In these strains the toxic antiphagocytic VW complex appears to be responsible for virulence Antibodies to the capsular antigen (Fraction 1) alone do not protect mice against infection with noncapsulated strains that contain VW However virulent strains as encountered in nature possess both capsular and VW antigens and at least in experimental infections of mouse rat and monkey antibodies to both antiphagocytic materials do not appear to be necessary for protection but only the anticapsular antibodies A maximum degree of protection may require both antibodies Discovery of the VW antigens provides an explanation for the older observations that although fully virulent wild type *P pestis* are encapsulated not all encapsulated strains are virulent It may be that the latter lack the VW antigens

2 *Nontoxic antigenic surface components which inhibit phagocytosis but antibodies to which are not protective* All pathogenic strains of *Bacillus anthracis* form capsules composed chiefly of D polyglutamic acid which has antiphagocytic properties However capsulated but nonvirulent strains exist as mentioned earlier The capsular material is antigenic but antibodies to it fail to protect animals against infection A protective antigen distinct from the polyglutamic acid capsule is produced during the course of infection and is present in edema fluid which can be used to immunize animals against the disease Under appropriate cultural con

ditions as shown by Gladstone (1946), the *protective antigen is formed in artificial culture medium* and this finding is the basis for the preparation of the immunizing materials currently in use for the protection of man (Wright *et al* 1954 Strange and Belton 1954) An exotoxin described by Smith and Keppie and their colleagues (see Smith and Keppie 1955) is lethal for laboratory animals and produces edema following injection into the skin It consists of 3 components called Factors I II and III each of which is a protein and each of which by itself is nontoxic A combination of I and II is both edema producing and lethal while addition of III to the combination results in a decrease in its capacity to provoke edema but in an increase in lethality (See Stanley and Smith 1961 1963 and Beall Taylor and Thorne 1962) Of the 3 factors II is the only one which when injected by itself into guinea pigs immunizes them against infection with *B anthracis* while addition of the other two alters the capacity of II to do so Preparations currently used for immunization of man against anthrax contain II and III The antiphagocytic polyglutamic acid capsule although uniformly possessed by virulent strains, is not a part of the protective antigen

3 *Nontoxic nonantigenic surface components which inhibit phagocytosis* Two examples may be cited the hyaluronic acid capsules of streptococci of Groups A and C and the fibrin coating derived from the host which is deposited on the surface of staphylococci that form coagulase

In addition to M proteins at the cell surface which on the basis of immunologic evidence are the primary determinants of pathogenicity, Group A streptococci form a second surface component hyaluronic acid that is also antiphagocytic though less active than the M proteins Hyaluronic acid is nonantigenic in man and other animals because similar or identical polymerized acidic polysaccharides are distributed throughout the animal body as constituents of the intercellular ground substance Evidence for the pathogenic role of hyaluronic acid in streptococci was obtained originally by Hirst (1941) who found that experimental infections of mice by Group C streptococci can be modified by injecting crude hyaluronidase preparations from leeches which act by depolymerizing the polysaccharide Hirst found no effect of hyaluronidase in Group A infections Subsequently Kass and Seastone (1944) using hyaluronidase prepared from bull testes reported a modifying effect on Group A infections of mice

For many years *coagulase* a product of most strains of pathogenic staphylococci was considered to be a virulence factor Coagulase is an enzyme or enzyme activator which in concert with a factor present in plasma of certain species, converts fibrinogen to fibrin It was held that coagulase acts by causing a fibrin coat to be deposited on the bacteria which protects them from phagocytosis (Smith Hale and Smith 1947) However, a primary role for coagulase in the pathogenicity of *Staphylococcus pyogenes* has not been proved At the present time it is more reasonable to consider coagulase production as one of the properties which characterize the species *Staphy pyogenes* along with many others such as production of golden pigment and α hemolysin ability to ferment mannitol and susceptibility to lysis by a variety of bacteriophages

4 *Toxic antigenic surface components (O antigens) of the enterobacteria and other gram negative bacteria* These substances termed endotoxins, are lipopolysaccharides whose role in infections and other pathologic conditions has been studied extensively Endotoxins are discussed in detail in a chapter which follows

5 *Exotoxic proteins which account for the principal pathogenic properties of bacteria* This category is exemplified by the classic exotoxins produced by *C diphtheriae Cl tetani* and *Cl botulinum* Injection into animals of the purified toxins in minute amounts reproduces the significant pathologic changes in each case and antibodies directed against the toxins are protective In none of these diseases is there invasion of the living tissues of the host indeed *Cl botulinum* should not be classed as an infectious agent The toxins of the 6 types of *Cl botulinum* are formed outside the animal body and, following ingestion are absorbed through the wall of the gut.

A brief description of the chemistry and the pharmacology of some important bacterial exotoxins is given in a subsequent section

6 *Extracellular products of bacteria that are not the principal pathogenic factors but may contribute to the disease picture* Examples which fall into this category are numerous In some instances the evidence is good for their participation in the natural disease in many there is as yet no solid evidence that they contribute to the pathologic changes In a third group such as the hyaluronidases of many bacterial species and the collagenase of *Cl welchii* although the experimental evidence would appear to rule them out as significant pathogenic factors doubt lingers as to whether or not it is adequate to exclude them entirely

Many of the substances in this category are extracellular enzymes that are produced during or shortly after active growth of the bacteria in vitro The manner in which they are released from the bacteria is not understood but may involve autolysis in some instances Some of them—for example the proteolytic enzyme of *Str pyogenes* and the ε toxin of *Cl welchii*—are released as inactive precursors that subsequently undergo conversion to active enzyme or toxin respectively The extracellular products are excreted during growth of the microorganisms also in natural disease as may be determined either by their pathologic effects or because specific antibodies to them appear during disease or in convalescence

Group A streptococci produce a greater number of extracellular toxic and enzymatic products than are recognized for most other human pathogens As many as 20 extracellular streptococcal antigens are detectable using normal pooled human gamma globulin as a source of antibodies (Halbert and Auerbach 1961) and only a minority of the antigens are identifiable with products of defined toxic or enzymatic activity Of these *erythrogenic* or *scarlatinal toxin* is responsible for the skin rash in scarlet fever which is essentially a streptococcal infection caused by a toxin producing strain in a person who has not acquired immunity to the toxin But erythrogenic toxin has no influence on the ability of streptococci to initiate infection so far as we are aware Its effect becomes apparent after invasion by streptococci has occurred Antibodies to the toxin which specifically neutralize its toxic action appear on recovery The manner in which erythrogenic toxin affects the small blood vessels to produce the scarlatinal blush is unknown

Most strains of hemolytic streptococci of Group A as well as some Group C and G strains produce an extracellular enzyme *streptokinase* which brings about digestion of fibrinogen and lysis of fibrin clots The remarkable specificity of streptokinase for fibrin was first described by Tillett and Garner (1933) who termed it fibrinolysin Subsequently it was shown by Christensen and MacLeod (1945) that it is a kinase which activates an enzyme precursor plasminogen in the plasma of man and animals to form the proteolytic enzyme plasmin Plasmin has affinity for fibrin and fibrinogen both in the test tube and in vivo and it seems likely that the thin exudates characteristic of streptococcal infections are due to the action of streptokinase At any rate as soon as the antibody antistreptokinase makes its appearance during the disease in man the exudates become thick and fibrinous presumably because antibody has neutralized the kinase It is not known whether streptokinase has other pathologic effects although both it and streptococcal hyaluronidase have been invoked to explain the typically rapid extension of streptococcal cellulitis through digestion of fibrin barriers and the intercellular ground substance Antistreptokinase does not prevent infection and information is not available that demonstrates whether it modifies the disease except as indicated above

In addition to erythrogenic toxin and streptokinase Group A streptococci produce two extracellular hemolysins or cytolysins streptolysin O and streptolysin S neither of which has been shown to take part in the pathogenesis of the infection although both are very powerful cytotoxins that cause death of animals when injected with very small doses Streptolysin O is released during infection as indicated by antibody production to it Streptolysin S in all probability is also excreted by the bacteria during disease but is not so easily detectable because either it

is not antigenic or else the antibody to it does not neutralize its toxic action

Streptolysin O is a protein which is readily oxidized to an inactive form by atmospheric oxygen It is related antigenically to oxygen labile hemolysins produced by pneumococcus (pneumolysin) *Cl tetani* (tetanolysin) and *Cl welchii* (θ toxin) Significantly none of the O hemolysins of these 3 species in common with streptolysin O has been shown to affect the course of natural or experimental disease

Certain strains of Group A streptococci produce an extracellular proteolytic enzyme of the papain type which is activated by KCN and sulfhydryl compounds The enzyme destroys M protein present on the surface of living cells and streptococci which produce large amounts are avirulent for mice Enhancement of virulence by mouse passage is accompanied by a diminished capacity of strains to produce the proteinase (Elliott 1945) Although production of the enzyme may affect the virulence of the bacterial cells themselves through digestion of M protein there is no evidence that its production in vivo is detrimental to the tissues of the infected host

Wilson (1957) has investigated the leukotoxicity of streptococci a property first described in 1918 by Levaditi This effect which is entirely distinct from the leukocidal action of the oxygen labile hemolysin streptolysin O occurs only after the bacteria are ingested and results in death of the phagocyte Filtrates of leukotoxic streptococci do not damage the phagocytes and a protective effect of antiserum cannot be demonstrated It appears that the leukotoxin is able to damage cells only if it is liberated within them and to this end the streptococci serve as Trojan horses Leukotoxin is produced by many but not all strains of Group A streptococci and its formation is associated especially with certain M types such as Type 12 The formation of leukotoxin is not correlated with mouse virulence Bernheimer Lazarides and Wilson (1957) have documented an interesting association between leukotoxicity and the production by certain strains of an enzyme streptococcal DPNase which destroys the coenzyme diphosphopyridine nucleotide The identity of leukotoxin and DPNase has not been proved As in the case of a number of other streptococcal extracellular products no correlation has been shown between their activity and virulence but as Wilson (1957) points out it is possible that in the contest between the invading bacteria and the cells of the host the leukotoxin might at times determine whether infection will be established or not The same might be said of other activities of Group A streptococci

Although apparently bearing no relationship to the phenomenon just described streptolysins O and S are known to destroy leukocytes in vitro (Todd 1942 Bernheimer and Schwartz 1960) Recently both of these agents have been shown to bring about the release of hydrolytic enzymes from lysosomes isolated from various tissues (Weissmann Keiser and Bernheimer 1963) In view of the lysosomal nature of the leukocytic granules it has been suggested that streptolysins O and S might disrupt the granules in intact white cells thereby releasing autolytic enzymes which in turn would digest other leukocyte structures resulting eventually in cell death Consistent with this concept is the finding that early rapid and extensive lysis of cytoplasmic granules does indeed occur when leukocytes are exposed to streptolysins (Hirsch Bernheimer and Weissmann 1963)

Cl welchii the most frequent cause of gas gangrene in common with other clostridia produces a powerful proteolytic enzyme *collagenase* which is capable of disintegrating muscle of laboratory animals in vivo by decomposing the reticular framework Pulping of affected muscles is a characteristic of clostridial myositis However it is by no means clear that collagenase contributes in a significant way to the pathogenesis of infection by *Cl welchii* Indeed the weight of evidence is against it since the specific antibody anticollagenase neither prevents infection nor appears to modify its course Similarly antibodies to *Cl welchii hyaluronidase* do not prevent infection nor do they mitigate the progress of the lesion Protection against infection depends on antibodies to the *α toxin* a *lecithinase* which hydrolyzes lecithin to phosphoryl choline and a diglyceride The lecithinase causes rapid lysis of erythrocytes

and necrosis of other cells and can account for most of the severe local effects following infection by *Cl welchii* However it is not proved that death from gas gangrene is caused by *α toxin absorbed from the involved* muscle and disseminated throughout the body Profound shock is seen in severe and fatal cases but free toxin is not demonstrable in the circulation nor does intravascular hemolysis occur except in cases of septic abortion in which in contrast with clostridial myositis the clostridia themselves are present in the blood

VARIATIONS IN VIRULENCE ASSOCIATED WITH THE PRODUCTION OF DIFFERENT AMOUNTS OF A PATHOGENIC FACTOR

Variations in virulence between strains of the same species or type may depend not only on the presence or the absence of a factor known to be concerned in pathogenicity but also on the amount produced For example in pneumococcus loss of capsulation through S ⟶ R mutation results in loss of virulence Mutants of intermediate virulence are commonly encountered and in strains of 3 different types it has been shown that virulence for mice is correlated with the amount of capsular polysaccharide produced *in vitro (MacLeod and Krauss 1950)* Studies by Wood (for summary see Wood 1951 52) explain in part the influence of different amounts of polysaccharide on virulence, since he has shown the pneumococci that possess large capsules are phagocyted with great difficulty Moreover if a strain produces large amounts of polysaccharide a lag in opsonization results because the host must produce larger amounts of specific antibody to combine with the excess of free polysaccharide in the tissues and body fluids as well as that present on the surface of the bacteria themselves The delay in opsonization permits more extensive multiplication Differences in capsule size corresponding to the amount of polysaccharide formed in vitro can be demonstrated in vivo In *Klebsiella pneumoniae* it has been found also that the amount of capsular polysaccharide and its rate of production bear a relationship to virulence (Ehrenworth and Baer, 1956)

A similar quantitative relationship may exist in diphtheria bacilli in which the exotoxin is the chief determinant of pathogenicity The simplest explanation of why *gravis* strains in general cause a more severe type of diphtheria than *mitis* strains would be that they are able to produce more toxin in vivo Pappenheimer and Johnston (1936) showed that the amount of iron present in the medium in which toxigenic diphtheria bacilli are growing determines the amount of toxin formed Under the conditions of their studies the amount of toxin increased as the iron concentration was increased up to 100 mcg of iron per liter which yielded peak toxin production As iron was increased beyond 100 micrograms per liter toxin production declined although growth improved At 500 mcg Fe per liter toxin production no longer could be demonstrated In explanation of the seemingly paradoxical relation between iron and toxin production it should be pointed out that whereas iron is essential for growth of the organisms no toxin is formed by the bacteria until the medium has been exhausted of iron (Mitsuhashi Kurokawa and Kojima 1949) At iron concentrations found optimal for toxin formation by laboratory strains no correlation has been found between toxin production of different strains isolated from patients and the severity of the disease they cause *Mueller (1941) observed that the* amount of iron present in diphtheritic membrane is many times that found to be optimal for toxin formation in vitro and that if a correlation exists between the amount of toxin and clinical severity it should be looked for under conditions where iron is in excess Mueller tested a *gravis* strain and 3 *mitis* strains under these conditions and found that in the presence of excess iron the *gravis* strain formed from 12 to 15 times as much toxin as the *mitis* strains Therefore it is tempting to relate differences in virulence of diphtheria bacilli to toxin production at high iron concentrations although this has not been shown to occur in vivo Pappenheimer (1955) has reported the growth rate of a *gravis* strain to be about 3 times as fast as a *mitis strain* the generation times being 60 and 160 minutes respectively If this difference in growth rate exists also in the diphtheritic membrane it might be expected that

is not antigenic or else the antibody to it does not neutralize its toxic action

Streptolysin O is a protein which is readily oxidized to an inactive form by atmospheric oxygen It is related antigenically to oxygen labile hemolysins produced by pneumococcus (pneumolysin) *Cl tetani* (tetanolysin) and *Cl welchii* (θ toxin) Significantly none of the O hemolysins of these 3 species in common with streptolysin O has been shown to affect the course of natural or experimental disease

Certain strains of Group A streptococci produce an extracellular proteolytic enzyme of the papain type which is activated by KCN and sulfhydryl compounds The enzyme destroys M protein present on the surface of living cells and streptococci which produce large amounts are avirulent for mice Enhancement of virulence by mouse passage is accompanied by a diminished capacity of strains to produce the proteinase (Elliott 1945) Although production of the enzyme may affect the virulence of the bacterial cells themselves through digestion of M protein there is no evidence that its production in vivo is detrimental to the tissues of the infected host

Wilson (1957) has investigated the leukotoxicity of streptococci a property first described in 1918 by Levaditi This effect which is entirely distinct from the leukocidal action of the oxygen labile hemolysin streptolysin O occurs only after the bacteria are ingested and results in death of the phagocyte Filtrates of leukotoxic streptococci do not damage the phagocytes and a protective effect of antiserum cannot be demonstrated It appears that the leukotoxin is able to damage cells only if it is liberated within them and to this end the streptococci serve as Trojan horses Leukotoxin is produced by many but not all strains of Group A streptococci, and its formation is associated especially with certain M types such as Type 12 The formation of leukotoxin is not correlated with mouse virulence Bernheimer Lazarides and Wilson (1957) have documented an interesting association between leukotoxicity and the production by certain strains of an enzyme streptococcal DPNase which destroys the coenzyme diphosphopyridine nucleotide The identity of leukotoxin and DPNase has not been proved As in the case of a number of other streptococcal extracellular products no correlation has been shown between their activity and virulence but as Wilson (1957) points out it is possible that in the contest between the invading bacteria and the cells of the host the leukotoxin might at times determine whether infection will be established or not The same might be said of other activities of Group A streptococci

Although apparently bearing no relationship to the phenomenon just described streptolysins O and S are known to destroy leukocytes in vitro (Todd 1942 Bernheimer and Schwartz 1960) Recently both of these agents have been shown to bring about the release of hydrolytic enzymes from lysosomes isolated from various tissues (Weissmann Keiser and Bernheimer 1963) In view of the lysosomal nature of the leukocytic granules it has been suggested that streptolysins O and S might disrupt the granules in intact white cells thereby releasing autolytic enzymes which in turn would digest other leukocyte structures resulting eventually in cell death Consistent with this concept is the finding that early rapid and extensive lysis of cytoplasmic granules does indeed occur when leukocytes are exposed to streptolysins (Hirsch Bernheimer and Weissmann 1963)

Cl welchii the most frequent cause of gas gangrene in common with other clostridia produces a powerful proteolytic enzyme *collagenase* which is capable of disintegrating muscle of laboratory animals *in vivo* by decomposing the reticular framework Pulping of affected muscles is a characteristic of clostridial myositis However it is by no means clear that collagenase contributes in a significant way to the pathogenesis of infection by *Cl welchii* Indeed the weight of evidence is against it since the specific antibody anticollagenase neither prevents infection nor appears to modify its course Similarly antibodies to *Cl welchii hyaluronidase* do not prevent infection nor do they mitigate the progress of the lesion Protection against infection depends on antibodies to the *α toxin* a *lecithinase* which hydrolyzes lecithin to phosphoryl choline and a diglyceride The lecithinase causes rapid lysis of erythrocytes

and necrosis of other cells and can account for most of the severe local effects following infection by *Cl welchu* However it is not proved that death from gas gangrene is caused by *α toxin absorbed from the involved muscle* and disseminated throughout the body Profound shock is seen in severe and fatal cases but free toxin is not demonstrable in the circulation nor does intravascular hemolysis occur except in cases of septic abortion in which in contrast with clostridial myositis the clostridia themselves are present in the blood

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ENHANCEMENT OF VIRULENCE

Enhancement of bacterial virulence is readily demonstrable experimentally on animal passage of bacterial strains For example most Group A streptococcal strains and some pneumococci on primary isolation from man are of low virulence for mice By repeated mouse passage virulence of pneumococcus may be increased from an LD_{50} of 100 000 bacteria or more to a degree where less than 10 cocci uniformly cause fatal infection Enhancement of virulence by animal passage results from selection of virulent mutants present in the original heterogeneous culture or that have arisen on multiplication in the animal body

It is commonly believed that during the course of epidemic disease in man the virulence of the infecting agent increases While this seems to be reasonable and may be true there is no objective evidence to support it The many factors involved in the spread of an epidemic disease make it almost impossible to establish that virulence has increased Perhaps the greatest difficulty in analysis lies in the fact that virulence for laboratory animals is rarely a measure of virulence for man and since virulence for man cannot be determined under accurately controlled conditions any inferences must be based on epidemiologic analysis Such analysis has not brought forth proof of increasing bacterial or viral virulence during the course of a human epidemic To the contrary it would appear more likely that a highly virulent and communicable mutant was selected through one means or another *before* the epidemic began If this were not the case it is difficult to visualize how an epidemic could start

A possible exception to the remarks in the preceding paragraph is the appearance of drug resistant mutants of bacteria under circumstances where large numbers of infected persons are treated over an extended period or where the drugs are used in low dosage for prophylaxis Under such circumstances sulfonamide resistant gonococci and Group A streptococci have appeared and have partially replaced the sufonamide susceptible strains previously encountered as the causes of epidemic disease of man There is no evidence that the resistant mutants are more virulent for *normal* man than susceptible strains However the additional attribute of drug fastness which enables resistant strains to cause disease in persons treated with sulfonamides, can perhaps be termed an enhancement of virulence since sulfonamide sensitive strains are not able to cause disease under the same circumstances

Virulence is also said to be increased by certain artificial methods such as the injection of bacteria suspended in mucin Enhancement of virulence by such methods should be regarded as more apparent than real Meningococci typhoid bacilli staphylococci and some other bacterial species are quite avirulent for mice and to cause death these animals must be injected in relatively enormous doses often 0 1 to 1 ml of broth culture The number of living organisms required to bring about a fatal infection does not differ markedly from the number of heat killed bacteria or their toxic products which cause death on injection If living meningococci or typhoid bacilli are suspended in a viscous protective medium such as mucin fatal infections result in mice after intraperitoneal injection of only a few bacteria The small inoculum protected by a surrounding coating of mucin presumably multiplies until sufficient bacteria are present to constitute a lethal toxic dose It may be added that studies of virulence in laboratory animals using bacteria suspended in mucin have provided little insight into the mechanisms involved in virulence and moreover have no relationship to virulence for man

Just as the virulence of bacteria may be increased by selection through repeated animal passage so it may often decrease by continued cultivation on artificial media The process again appears to be one of selection in this case of *less* virulent mutants Many examples of such loss of virulence on laboratory media following isolation might be given of which only one will be discussed

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CHEMISTRY AND PHARMACOLOGY OF BACTERIAL TOXINS

Classic Exotoxins of Gram Positive Bacteria

Several examples of diffusible toxins pro duced by gram positive bacteria have been referred to already These substances have been called exotoxins because they are found in filtrates of growing organisms exhibiting no visible evidence of autolysis or else are found to increase in amount parallel with growth In some instances such as *Cl botu linum* and *Cl tetani* the yield of toxin is increased on autolysis Exotoxins are charac teristic of gram positive bacteria and are produced by many pathogenic species Gram negative bacteria on the other hand produce lipopolysaccharide endotoxins which form a part of the O or somatic antigen and are intimately associated with the structural in tegrity of the cells They are not liberated into the medium unless autolysis has oc curred An exception among gram negative bacteria is the type species *Shigella dysen teriae* that produces a very potent protein exotoxin the so-called shiga neurotoxin dis tinct from the endotoxin Antibodies to the neurotoxin neutralize its toxicity but do not

TABLE 2 AMINO ACID COMPOSITION OF TETANUS AND BOTULINUS TOXINS AND OF RABBIT α GLOBULIN (ANTIBODY TO PNEUMOCOCCAL POLYSACCHARIDES)

	Tetanus Toxin[1]	Botulinus Toxin Type A	Rabbit[3] Globulin
	per cent	per cent	per cent
Nitrogen	15 7	16 3	16 0
Sulfur	1 04	0 44	1 32
Cystine	—	0 53	2 25
Cysteine	—	0 27	—
Methionine	1 78	1 06	1 40
Tyrosine	6 47[4]	13 5	6 62
Tryptophane	0 91	1 86	2 70
Phenylalanine	4 91	1 17	5 49
Arginine	3 36	4 62	5 04
Histidine	1 15	1 03	1 53
Lysine	10 0	7 74	6 46
Aspartic acid	15 3	20 26	9 67
Glutamic acid	10 3	15 57	11 8
Isoleucine	9 36	11 94	4 39
Leucine	8 23	10 30	7 90
Threonine	5 13	8 49	13 22
Valine	5 39	5 29	10 22
Glycine	3 34	1 38	5 72
Alanine	4 09[4]	3 92	5 68
Serine	5 27[4]	4 36	10 92
Proline	3 66[4]	2 60	8 56
Sedimentation constant	4 5S	17 3S	6 3S
Molecular weight	67 000	900 000	160 000

[1] Dunn M S Camien M N and Pillemer L Arch Biochem 1949 *22* 374-376

[2] Buehler H J Schantz E J and Lamanna C J Biol Chem 1947 *169* 295 302

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Phenylalanine	4 91	1 17	5 49
Arginine	3 36	4 62	5 04
Histidine	*1 15*	*1 03*	*1 53*
Lysine	10 0	7 74	6 46
Aspartic acid	15 3	20 26	9 67
Glutamic acid	10 3	15 57	11 8
Isoleucine	9 36	11 94	4 39
Leucine	8 23	10 30	7 90
Threonine	5 13	8 49	13 22
Valine	5 39	5 29	10 22
Glycine	3 34	1 38	5 72
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have been obtained in crystalline form (Abrams *et al* 1946 Lamanna *et al* 1946) These proteins in common with shiga neurotoxin are among the most powerful poisons known It has been estimated that 1 mg of crystalline tetanus or botulinus toxin is sufficient to kill 1 000 tons of guinea pig The minimal lethal dose of botulinus toxin for the mouse is only 20 000 000 molecules Different animal species show great variation in susceptibility to bacterial toxins (Metchnikoff 1905) Man horse and guinea pig are extremely susceptible to tetanus botulinus and diphtheria toxin The mouse and the rat are only about 1/1000th as sensitive as the guinea pig to diphtheria toxin The dog while very resistant to botulinus and tetanus toxins is highly sensitive to diphtheria toxin Rabbits are approximately 10 000 times as susceptible to the killing action of shiga neurotoxin as guinea pigs

Cold blooded animals are completely resistant to large doses of tetanus botulinus and diphtheria toxins with the interesting exception of the frog and certain lizards Injection of tetanus toxin into these amphibia has no effect at low temperatures but symptoms of tetanus appear when the creatures are kept at temperatures above 20 C

On the basis of the amount of purified toxin required to produce death (LD_{50}) in a susceptible species such as the guinea pig tetanus and botulinus toxin the most poisonous substances known are about 400 times as toxic as diphtheria toxin The reason for the difference may lie in the fact that tetanus and botulinus toxins have selective affinity and toxicity for limited portions of the nervous system whereas diphtheria toxin is a general poison that is taken up by and affects any cells it comes in contact with

Chemical analysis of bacterial toxins has not provided an explanation for their extraordinary toxicity Reasonably complete information on amino acid composition of tetanus and type A botulinus toxin is available as shown in Table 2 in which for the sake of comparison data are presented for a well characterized protein rabbit γ globulin antibody to pneumococcal polysaccharides No unusual chemical groupings that can account for toxicity have been discovered Dunn *et al* (1949) have noted that in tetanus and type A botulinus toxins the ratios of aspartic acid to glutamic acid and of isoleucine to leucine are reversed as compared with most other proteins that have been studied Toxicity appears to depend on the spatial configuration of amino acids and peptide chains within the intact protein molecule and procedures which alter the protein are likely to cause loss of toxicity More recent analyses of tetanus toxin diphtheria toxin staphylococcal α toxin and the ε prototoxin of Type D *Cl welchii* indicate that these do not differ substantially in amino acid composition from the total bacterial protein

Toxoid Formation

A variety of agents which react with bacterial toxins cause irreversible loss of toxicity without loss of antigenicity or of power to combine with antitoxin All of these reagents apparently attack free amino groups they include iodine ketene (an acetylating agent) and diazonium salts However, the most commonly used reagent is dilute formaldehyde It was discovered by Glenny and Hopkins and by Ramon that crude diphtheria toxin could be completely detoxified by treatment with dilute formalin (0 4 0 5%) at slightly alkaline pH The reaction is usually complete after 3 or 4 weeks at 37 C The detoxified product termed *anatoxine* by Ramon (1928) and *toxoid* by Glenny and Hopkins (1923) retains its immunologic specificity and antigenic properties Tetanus and botulinus toxoids may be prepared in a similar manner by treatment of the corresponding toxins with formalin Diphtheria and tetanus toxoid are used on a large scale for active immunization of man against diphtheria and tetanus

The change from toxin to toxoid is a property common to most bacterial toxins and is a process which can occur spontaneously to a certain extent even at low temperatures This spontaneous detoxification without parallel loss in immunologic combining power was observed by Paul Ehrlich in 1903 who was in fact the first to use the term toxoid

Pharmacologic Action of Diphtheria Tetanus and Botulinus Toxins and Lecithinase of Cl welchii

The pharmacologic action of each of these toxins is different Diphtheria toxin causes damage to almost all types of cell in the sus

ceptible animal whereas the action of botulinus toxin is restricted to cholinergic nerve endings in peripheral autonomic and somatic fibres and tetanus toxin appears to affect only the motor neurons in the cerebrospinal axis As noted above the selectivity of action of tetanus and botulinus toxins can well account for their extraordinary toxic effect as measured by their LD_{50}, in susceptible animals The α toxin of *Cl welchii* is a lecithinase

Pappenheimer and his associates (for review see Pappenheimer 1955) have accumulated suggestive evidence that the toxity of diphtheria toxin is due to its interference with the cytochrome system of the cells of susceptible animals Toxin is formed by diphtheria bacillus only in the presence of oxygen and a supply of iron less than is necessary for optimal growth In studies of the Park Williams 8 strain which is used in most laboratories for toxin production it has been shown that bacteria harvested from cultures in which an amount of iron optimal for toxin formation is present have a very low iron content and that all iron-containing respiratory enzymes are reduced in amount This is particularly striking in the case of cytochrome b_1 the principal respiratory pigment to which 9/10ths of the hemin iron is bound As toxin is produced by iron depleted cells coproporphyrin III is also excreted into the medium The ratio of toxin to porphyrin is 1 4 the same as is present in iron-containing respiratory enzymes This observation suggested to Pappenheimer that toxin may be related to the protein portion of diphtherial cytochrome b_1 and that toxin may cause injury to the cells of susceptible animals by interfering with cytochrome b a respiratory pigment which is similar to diphtherial cytochrome b_1 and like it is concerned in succinate oxidation This ingenious scheme to account for the toxicity of diphtheria toxin has not yet been proved but strong indirect evidence for it has been presented

Macfarlane and Datta (1954) have shown that succinoxidase activity of mitochondria is reduced markedly by the α toxin (lecithinase) of *Cl welchii* The mechanism is distinct from that proposed for diphtheria toxin and is associated with hydrolysis of lecithin in the mitochondria (for discussion see review by Macfarlane 1955) Macfarlane points out that the decrease in the oxidative processes of the cells causes a shift in metabolism to glycolysis When this occurs the accumulation of organic acids would further damage the cells of the host Lecithinase of *Cl welchii* is also strongly hemolytic in vitro or on intravenous injection in animals because of digestion of lecithin of the red cell membrane However hemolysis is not a feature of the local infection clostridial myositis but does occur often to a marked degree in the bacteremia of septic abortions in which *Cl welchii* is the infecting agent The genesis of the profound shock and death seen in severe clostridial myositis is unknown Because there is no evidence of hemolysis it has been suggested that the general effects are due not to toxin carried throughout the body via the bloodstream but to some other product elaborated in the affected muscle

In contrast with the action of diphtheria toxin and lecithinase of *Cl welchii* botulinus and tetanus toxins act only on the nervous system (for review see Wright 1955) The parallelism between the sites of action of acetylcholine and botulinus toxin made it appear probable that botulinus toxin has an anticholinergic action and it has been shown that the toxin acts very widely on all parts of the peripheral nervous system that are cholinergic whether they are preganglionic or postganglionic components of the autonomic system or are the motor innervation of skeletal muscles The studies of Burgen Dickens and Zatman (1949) demonstrated that botulinus toxin depresses the release of acetylcholine and the pharmacologic effects can be explained best on this basis

Whereas botulinus toxin acts on peripheral nerves the action of tetanus toxin is in the cerebrospinal axis only and there is no clear evidence that it has any action on peripheral nerves From its site of introduction tetanus toxin travels to the central nervous system along peripheral nerves Early studies suggested travel within the axon itself but the later work of Baylis and his colleagues (1952) indicates strongly that the toxin moves centripetally in the interneuronal tissue spaces propelled by the pressure from contracting muscles and enters the interstitial tissue fluid of the central nervous system at the point of emergence of the peripheral nerve trunks from the spinal cord Its effect

have been obtained in crystalline form (Abrams *et al* 1946 Lamanna *et al* 1946) These proteins in common with shiga neurotoxin are among the most powerful poisons known It has been estimated that 1 mg of crystalline tetanus or botulinus toxin is sufficient to kill 1 000 tons of guinea pig The minimal lethal dose of botulinus toxin for the mouse is only 20 000 000 molecules Different animal species show great variation in susceptibility to bacterial toxins (Metchnikoff 1905) Man horse and guinea pig are extremely susceptible to tetanus botulinus and diphtheria toxin The mouse and the rat are only about 1/1000th as sensitive as the guinea pig to diphtheria toxin The dog while very resistant to botulinus and tetanus toxins is highly sensitive to diphtheria toxin Rabbits are approximately 10 000 times as susceptible to the killing action of shiga neurotoxin as guinea pigs

Cold blooded animals are completely resistant to large doses of tetanus botulinus and diphtheria toxins with the interesting exception of the frog and certain lizards Injection of tetanus toxin into these amphibia has no effect at low temperatures but symptoms of tetanus appear when the creatures are kept at temperatures above 20 C

On the basis of the amount of purified toxin required to produce death (LD_{50}) in a susceptible species such as the guinea pig tetanus and botulinus toxin the most poisonous substances known are about 400 times as toxic as diphtheria toxin The reason for the difference may lie in the fact that tetanus and botulinus toxins have selective affinity and toxicity for limited portions of the nervous system whereas diphtheria toxin is a general poison that is taken up by and affects any cells it comes in contact with

Chemical analysis of bacterial toxins has not provided an explanation for their extraordinary toxicity Reasonably complete information on amino acid composition of tetanus and type A botulinus toxin is available as shown in Table 2 in which for the sake of comparison data are presented for a well characterized protein rabbit γ globulin antibody to pneumococcal polysaccharides No unusual chemical groupings that can account for toxicity have been discovered Dunn *et al* (1949) have noted that in tetanus and type A botulinus toxins the ratios of aspartic acid to glutamic acid and of isoleucine to leucine are reversed as compared with most other proteins that have been studied Toxicity appears to depend on the spatial configuration of amino acids and peptide chains within the intact protein molecule and procedures which alter the protein are likely to cause loss of toxicity More recent analyses of tetanus toxin diphtheria toxin staphylococcal α toxin and the ε prototoxin of Type D *Cl welchii* indicate that these do not differ substantially in amino acid composition from the total bacterial protein

Toxoid Formation

A variety of agents which react with bacterial toxins cause irreversible loss of toxicity without loss of antigenicity or of power to combine with antitoxin All of these reagents apparently attack free amino groups they include iodine ketene (an acetylating agent) and diazonium salts However, the most commonly used reagent is dilute formaldehyde It was discovered by Glenny and Hopkins and by Ramon that crude diphtheria toxin could be completely detoxified by treatment with dilute formalin (0 4-0 5%) at slightly alkaline pH The reaction is usually complete after 3 or 4 weeks at 37 C The detoxified product termed *anatoxine* by Ramon (1928) and *toxoid* by Glenny and Hopkins (1923) retains its immunologic specificity and antigenic properties Tetanus and botulinus toxoids may be prepared in a similar manner by treatment of the corresponding toxins with formalin Diphtheria and tetanus toxoid are used on a large scale for active immunization of man against diphtheria and tetanus

The change from toxin to toxoid is a property common to most bacterial toxins and is a process which can occur spontaneously to a certain extent even at low temperatures This spontaneous detoxification without parallel loss in immunologic combining power was observed by Paul Ehrlich in 1903 who was in fact the first to use the term toxoid

Pharmacologic Action of Diphtheria Tetanus and Botulinus Toxins and Lecithinase of Cl welchii

The pharmacologic action of each of these toxins is different Diphtheria toxin causes damage to almost all types of cell in the sus

organisms has lagged far behind knowledge of factors which influence virulence

Although communicability must be possessed by naturally pathogenic species it is not confined to virulent microorganisms The bacteria which comprise the normal flora of the skin the oropharynx the alimentary tract and the external genitalia of healthy persons are by definition communicable even though they rarely cause disease in normal persons Indeed certain of the microorganisms forming the normal flora of the alimentary canal may be essential for health through the synthesis of vitamins or the partial digestion of nutrients for which the host does not possess enzymes For example much of the vitamin K requirement of man and animals appears to be supplied by intestinal bacteria which are able to synthesize it In ruminants bacteria in the stomachs play a very important part in the digestion of food

In contrast with microorganisms that are communicable but nonvirulent are those that are virulent when introduced into the animal body but are not communicable from experimentally infected animals to normal cage mates For example pneumococci are virulent when introduced experimentally into mice by almost any route One or two cocci of the most virulent strains will cause the death of mice following intraperitoneal injection but normal mice kept in the same cage remain well

Because communicability and virulence are not necessarily interdependent it would appear that epidemic strains of a bacterial species are those in which natural selection has operated to permit the survival of variants which are concurrently highly communicable and virulent

The site of the lesions in the infected host has an important influence on communicability and this may have a bearing on the noncommunicability of pneumococcal infections in mice where the disease does not involve the respiratory tract primarily even though the bacteria are placed directly within the trachea but instead produces a rapidly fatal bacteremic infection If the disease were localized in the lungs and mice had the capacity to cough and spit pneumococcal infection might occur as an epizootic disease in this species

The influence of the site of the lesion on communicability is illustrated by plague in man which occurs in two well recognized clinical forms called bubonic and pneumonic plague The bubonic form is spread from rats to man through the bite of the rat flea Buboes form in areas adjacent to the point where the organisms are introduced through the bite of the flea and although invasion of the bloodstream commonly occurs secondary cases of plague do not arise because the bubonic form is essentially a closed infection On the other hand pneumonic plague caused by the same bacterial species *Pasteurella pestis* is contagious to those in proximity to the sufferer who expels enormous numbers of plague bacilli during coughing and in the copious discharges from the respiratory tract Secondary cases of pneumonic plague occur under certain undefined environmental circumstances because the open nature of the pulmonary lesions permits dissemination of the bacilli

A less exotic illustration of the influence of the site of the lesions on the communicability of bacteria is afforded by the hemolytic streptococcus The expression dangerous carrier has come into current usage to indicate infected individuals from whom secondary cases arise commonly Several independent investigations have shown that patients with purulent lesions discharging on the body surface as for example otitis media cervical adenitis and wound infections are more likely to transmit infection than patients with uncomplicated streptococcal pharyngitis The reason for this difference in communicability from different lesions caused by the same streptococcal strains may be because purulent lesions discharge more streptococci into the environment contaminating heavily everything in contact with the patient whereas from patients with uncomplicated pharyngitis fewer organisms are discharged into the environment therefore there is less chance of secondary cases arising

Size of the Inoculum In the case of virulence of bacteria the size of the inoculum is of great significance The same may also be true of communicability though this has not been demonstrated When a microorganism

is on motor neurons exclusively Ambache (1948) and his associates have demonstrated in local tetanus intoxication of the eye that the formation of acetylcholine is greatly reduced There is evidence therefore that both botulinus and tetanus toxin act through interference with cholinergic mechanisms and that the mode of action in each case may be similar The great difference in the action of the toxins is that tetanus acts centrally in the cerebrospinal axis whereas botulinus toxin affects only cholinergic nerve endings in peripheral somatic and autonomic fibers The degeneration of anterior horn cells observed in tetanus intoxication is believed to be a secondary manifestation and is not the cause of the characteristic symptomatology

It has been known since the 19th century that nervous tissue binds or fixes tetanus toxin in vitro In this respect brain tissue is more effective than spinal cord and gray matter more than white The constituent responsible for fixation of the toxin has been identified as ganglioside (van Heyningen 1959 van Heyningen and Miller 1961) and the capacity of ganglioside to form a complex with toxin depends in part on its content of sialic acid whose free carboxyl group is essential for fixation It is interesting and perhaps significant that ganglioside also fixes strychnine and certain other drugs which by suppressing synaptic inhibition have the same neurophysiologic action as tetanus toxin (van Heyningen 1963) Interaction of toxin with ganglioside does not appear to bring about any enzymatic alteration of this constituent of nervous tissue and hence the precise manner in which combination of toxin with ganglioside may affect motor neurons remains to be elucidated The low order of sensitivity of the frog to tetanus toxin can be attributed to the fact that frog nervous tissue has very little capacity to fix toxin (van Heyningen and Woodman 1963)

THE RELATION OF HYPERSENSITIVITY TO DISEASE PROCESSES

Although hypersensitivity to components of the bacterial cell undoubtedly plays a considerable part in the type and the progress of the lesions in many infectious diseases especially those of chronic nature little specific information is available except in the case of tuberculosis In tuberculosis in guinea pigs the effect of hypersensitivity to tuberculoproteins which in themselves are non toxic can be demonstrated readily Normal guinea pigs injected with as much as 2 ml of Koch s Old Tuberculin do not suffer any obvious ill effects On the other hand if a tuberculous guinea pig during the 8th or the 10th week of infection is injected with as little as 0 01 ml of tuberculin death may occur within a few hours An intense inflammatory reaction occurs at the site of injection and throughout the body wherever tubercles are present

The generalized tuberculin reaction in the guinea pig which ends in death illustrates the ill effects of the allergic reaction in its most severe form Less spectacular but nonetheless damaging effects due to the hypersensitive state that uniformly exists and the presence in the lesions of tubercle bacilli and their products almost certainly occur in the course of the tuberculous process in man and animals It is likely that the destructiveness of the tuberculous lesions in the adult type of tuberculosis with extensive caseation and fibrosis is strongly influenced by hypersensitivity

In other chronic bacterial infections for example brucellosis hypersensitivity to the bacteria and their products likewise may have an important influence on the character and the persistence of the lesions However this has not been clearly defined Up to the present time there is no good evidence that the hypersensitive state is part of the host s defense against infection

THE COMMUNICABILITY OF BACTERIA

A great deal more is known of the factors influencing the pathogenicity of micro-organisms than of the properties they must possess in order to be communicable from host to host under natural conditions Pathogenicity is more susceptible to experimental study because of its end result—disease—whereas organisms may be transmitted from host to host without any detectable pathologic alteration For this reason the study of properties concerned in communicability of micro

control of infectious disease by quarantine of the sick for example would be a relatively simple procedure However since perfectly well individuals either recovered from infection or who at no time have shown clinical evidence of disease may harbor the fully virulent pathogen it is apparent that for most infectious diseases quarantine of the sick is not an effective control procedure

The development of a carrier state in normal individuals who at no time have shown evidence of disease is of more significance in many instances than the persistence of the agent in recovered patients This is true in the case of meningococci for example where the number of normal persons who carry the microorganism exceeds by far those who develop meningococcal infection In this instance the person who never has been ill is of greater importance in the transmission of the microorganisms than those recovered from infection With the typhoid bacillus on the other hand the individual who has recovered from the disease is the primary source for the maintenance and the dissemination of the bacteria Therefore it is apparent that no general rule can be laid down concerning the circumstances under which pathogens can best survive in the body With different microorganisms affecting different areas of the body the conditions of parasitism vary

The ability to survive outside the animal body in which disease is produced or in which they can be carried intermittently may have considerable significance in the communicability of many bacteria The most striking illustrations of organisms having the ability to survive outside the animal body are the spore forming pathogens Anthrax spores may survive in pasture land for as long as 12 years as described by Pasteur in 1881 and animals feeding on it may be infected The spores of the tetanus bacillus and the anaerobic bacteria associated with gas gangrene which find their way to the soil especially through the feces of man and animals survive there for long periods of time and on introduction into wounds may germinate and cause disease In the case of organisms existing only in a vegetative phase the capacity to survive outside the animal body is not nearly so great as for spore formers although if vegetative forms are dried rapidly they may remain viable for long periods of time under natural climatic conditions

Numerous studies of the environment have been made in an attempt to explain the epidemiology of hemolytic streptococcal infections of the respiratory tract In places where the disease is endemic living streptococci can be isolated from clothing bedding and other articles as well as from dust and from the air itself The general contamination of the environment was long considered as being of great significance in the spread of streptococcal disease and based on this circumstantial evidence elaborate procedures were set up in military establishments to reduce it Oiling of floors to hold down dust borne streptococci had no influence on the incidence of disease nor did sterilization of air by glycol aerosols or ultraviolet light The studies of Rammelkamp Wannamaker Perry and their colleagues (for review see Rammelkamp 1955 56) have explained why such procedures are ineffective They have presented strong evidence that transmission is direct from man to man and does not appear to occur commonly at a distance through the air or because of dried streptococci present in the dust or on fomites Direct contact with fresh moist secretions of the infected person appears to be the important means of transmission Indeed these investigators have demonstrated that living but dried Group A streptococci present in floor dust are not infectious for man even when instilled into the nasopharynx in large numbers It would appear likely that they are taken up and destroyed by phagocytic cells during the prolonged lag phase before growth can be initiated from the dried state

Likewise pneumococci have been isolated from the environment in places where pneumococcal pneumonia was epidemic and it has been assumed that this environmental contamination is of importance in spread Similarly in the case of epidemic staphylococcal infections in hospitals which constitute such a serious problem at the present time airborne spread is considered as being of great importance although no solid evidence is available to prove it

Unless it is shown otherwise it would ap

TABLE 3 OPERATION OF CHANCE IN PNEUMOCOCCAL CARRIER STATE

Number of carriers of	
1 pneumococcal type	1 317
2 pneumococcal types	200
3 pneumococcal types	27
4 pneumococcal types	4
Ratio of carriers of	
2 types to carriers of 1 type	$\frac{200}{1\,317} = 0\,152$
3 types to carriers of 2 types	$\frac{27}{200} = 0\,135$
4 types to carriers of 3 types	$\frac{4}{27} = 0\,148$

is transmitted from one individual of a species to another of the same species com municable variants alone are involved pre sumably because only these variants would have survived in the original host The size of the inoculum here should play only a small part since most of the bacterial popu lation would consist of communicable vari ants However in transmission from one species of animal to another it is probable that the size of the inoculum is of consider able significance because the conditions nec essary for survival in the new host are likely to be different from those in the original host if the inoculum is large there is more chance of communicable mutants being present than if it is small

Chance In transmission from one host to another of the same species chance may determine largely whether or not the new host becomes a carrier although even in individuals of the same species there may be differences which influence the capacity to become carriers By chance is meant simply the accident of coming in contact with a bacterial species or strain The operation of chance is well illustrated in the communi cability for man of pneumococci (Hodges and MacLeod 1946) From 40 to 70 per cent of normal adult humans carry one or more of the many serologic types of pneumo cocci in the pharynx at any given time some persons carrying as many as 5 distinct types all at once The ratios of carriers of 2 types to 1 type 3 types to 2 types etc as shown in Table 3 are approximately the same indicating that in the main chance has determined how many types of these communicable organisms are carried by an individual

However further analysis of pneumo coccal carriers shows that host factors also operate in determining whether or not an individual will become a carrier of a particular pneumococcal type (MacLeod Hodges Heidelberger and Bernhard 1945) In a population of which half the members were immunized against pneumococcus Types 1 2 5 and 7 it was found that sig nificantly fewer of the immunized men were carriers of these types as compared with the nonimmunes It is apparent therefore that pneumococci are not as communicable to immune persons as to nonimmunes Anal ogous data are not available for other bacteria although a similar state of affairs probably exists for diphtheria bacilli also In this case immunization of approximately half of the susceptible population affords a very considerable measure of protection to the nonimmunized portion as was found true also for pneumococcal infection in a partially immunized population The chain of transmission from one susceptible indi vidual to another appears to be broken by the interposition of immune individuals who are less able to act as carriers In other words the immune status of the individual in the case of pneumococci and probably diphtheria bacilli exerts a powerful influence on communicability

Survival Capacity in Immune Subjects Whereas with certain bacteria the immune individual is less likely to become or remain a carrier than the nonimmune the capacity of many bacterial species to survive in im mune subjects is an important contributing factor in communicability An obvious ex ample is the persistence of typhoid bacilli in the biliary tracts of a proportion of those who have recovered from the disease whence the organisms are discharged into the intes tinal canal and through subsequent fecal contamination of food or water become transmitted to susceptible individuals If the reservoir of infectious agents consisted only of persons actually sick with infection the

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pear more reasonable to consider that when bacteria in the dried state are present as general contaminants of the environment this may have no more significance than as a reflection of the fact that disease caused by them is taking place in that environment It should not be assumed that these dried environmental deposits have a significant part to play in spread

Survival in water has an important bearing on communicability of enteric pathogens such as those causing typhoid or paratyphoid fevers dysentery and cholera, all of which may be water borne diseases though not transmitted in this manner exclusively

The ability to survive and multiply in an intermediate host or vector is of prime significance with some microorganisms, of which plague is a good example The plague bacillus causes natural disease in many species of rodents in different parts of the world and is transmitted to man through the bite of various species of fleas which are ectoparasites of the rodents The bacilli are able to multiply and cause disease in the upper portion of the alimentary tract of the fleas which derive their infection from the rodent reservoir The ability of plague bacilli to infect fleas is thus a crucial factor in their communicability *Pasteurella tularense* an organism related to the plague bacillus and the cause of tularemia has its reservoir also in various rodent species and may be carried by arthropods such as ticks and deer flies which feed on the rodents Although in the United States the disease in man is most often acquired during the skinning and the dressing of wild rabbits it is also possible for transmission to occur through the bite of the arthropod vectors

Flies (*Musca domestica*) have long been suspected as being of importance in the transmission of typhoid fever In this instance however the insect occupies a relatively passive role in that the fly is not infected by the typhoid bacillus, nor does it become a permanent carrier of the microorganisms The feet of the fly and other parts of its body surface become contaminated during contact with feces and then the organisms may be transferred mechanically to human food

From this discussion it can be seen that certain of the general properties necessary for communicability of various bacterial species can be defined However as noted earlier little information of a specific nature is available With pathogenic species the communicable organisms carried in the body usually possess the cell structures known to be associated with virulence though this is not uniformly true Pneumococci isolated from normal humans are in the encapsulated that is potentially pathogenic state and the nonencapsulated rough variants are not found On the other hand, hemolytic streptococci isolated from carriers not infrequently have lost the M protein which is known to be concerned in their virulence Therefore these organisms must possess a component or components other than the important M protein which prevent their destruction by the body It can be argued that such avirulent variants, lacking M protein may have no pathogenic significance However, on transmission to a nonimmune host the capacity to produce M protein may be regained because of mutation or possibly as a result of genetic recombination through mechanisms such as transduction or transformation

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by the wall of fibrin deposited about many abscesses

Thus anatomic barriers commonly are considered to serve in host defense by limiting penetration and dissemination of microorganisms In some situations however structures within the body appear to permit passage of microbes but not phagocytic cells thus offering havens in which bacteria are protected from usually effective cellular host defense systems Consider for example the pathogenesis of subacute bacterial endocarditis The microbe involved in this severe or even fatal disease is commonly an alpha hemolytic streptococcus otherwise an essentially avirulent organism Alpha streptococci can be introduced into the tissues or even the bloodstream of the normal person with production of little or no disease but in the rare individual with a congenital anomaly of the large blood vessels or with endocardial scars from previous rheumatic fever a few alpha hemolytic streptococci may alight on these abnormal areas and multiply locally to produce the typical lesions called vegetations Microbes disseminated to other tissues from these vegetations are promptly ingested by phagocytes and destroyed but apparently the anatomic arrangements within the vegetation itself afford shelter for the streptococci from host defense mechanisms

Similarly a piece of devitalized bone a sequestrum may provide for staphylococci or other organisms causing osteomyelitis a focus which cannot be penetrated by phagocytic cells because of the microanatomy and such a situation usually leads to persistent infectious disease

Antibody and Phagocytes

Antibody complement and phagocytic cells usually are considered to be the main agencies by which the host rids itself of invading microbes These systems will be discussed in detail in following chapters therefore they are mentioned here only for continuity and for a few general comments Humoral and cellular mechanisms for combating invading microbes or their toxins were discovered at approximately the same time (1890) and together formed the corner stone for the science of immunology For decades a polemic raged between workers led by Von Behring and Pfeiffer who claimed that humoral factors were mainly responsible for host resistence to infectious diseases and scientists under Metchnikoff at the Pasteur Institute who proposed on the contrary that such resistance was due mainly to the action of phagocytic cells Signs of this argument still remain but the debate now has been largely resolved in favor of *both* sides Most types of microbes survive exposure to serum even in the presence of specific antibody Invading bacteria by and large are killed following engulfment by phagocytic cells However phagocytes are able to engulf bacteria efficiently only if serum factors (opsonin and/or antibody) are present Antibodies also clearly play a central role in neutralizing microbial exotoxins Thus effective host resistance requires conjoint action of both humoral and cellular factors

The crucial role of antibodies and of phagocytic cells in resistance to infectious diseases is established beyond doubt The enormous increase in susceptibility to sepsis of persons lacking antibodies (agammaglobulinemia) or lacking phagocytes (agranulocytosis) illustrates clearly the importance of these factors in host defense However much remains to be learned about their precise mechanism of action For instance much is known about the nature of antibodies in terms of their site of formation and chemical properties but little can be said concerning the intimate nature of their action Similarly although we know much about morphologic and other alterations accompanying the killing of bacteria within phagocytes the substance or system responsible for the bactericidal effect remains in doubt or in some cases is completely unknown

The Chemical Microenvironment

Many phenomena indicate that host resistance is based at least in part on factors other than anatomic barriers antibodies and phagocytes One such phenomenon has to do with localization of bacterial infectious diseases Most microbes show a distinct preference for multiplication in a certain tissue or organ of a given host For example streptococci pneumococci and meningococci usually

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8

Host Resistance to Infectious Disease

*In modern times endemic patterns of in*fectious diseases are prevalent in which the causative microbes may be widely disseminated in the population but only rarely produce detectable disease For example fully virulent forms of staphylococci pneumococci meningococci diphtheria and certain clostridia are found frequently in cultures taken from skin mucous membranes or intestinal contents of healthy persons In this setting the critical determinant of disease is not merely exposure or contact but rather the net result of the complex interaction between virulence factors of the microbes and resistance mechanisms of the host

It is our aim in this chapter to consider from the over all point of view some of the general and the specific mechanisms involved in host resistance to bacterial infectious diseases

BASIC MECHANISMS OF HOST RESISTANCE

Anatomic Barriers

Perhaps the most obvious of the host resistance mechanisms is that of barriers posed by skin mucous membranes and other anatomic structures The surface linings of the intestines the upper respiratory tract and the skin in the normal state are swarming with untold numbers of microorganisms whereas beneath these surfaces in the submucosa or the subcutaneous tissue sterile conditions usually prevail microbes on the surface are unable to pass freely through these anatomic barriers

That such barriers do in fact function to protect the host against infectious disease is obvious Everyone knows that skin lacerations commonly are followed by local infectious disease or that a perforated appendix leads to peritonitis However, recent observations indicate that even in the healthy person skin and mucous membranes are penetrated relatively frequently by small numbers of bacteria Minute abrasions accompany such everyday activities as chewing and digesting food brushing teeth shaving or scratching the skin and so forth Apparently tiny breaks in the barrier permit passage of the surface microorganisms for if cultures are taken sufficiently frequently bacteria of the normal intestinal or skin flora can be recovered on occasions from tissues regional lymph nodes and even from the bloodstream (see Hirsch 1960) This invasion by bacteria probably occurs several times daily in everyone but the small numbers of relatively nonvirulent organisms so introduced are eliminated readily by other host defense mechanisms and no disease ensues

In addition to skin and mucosal barriers there exist within the tissues other membranes such as muscle fascia joint synovia or pleural surfaces which act in many instances to localize an infectious process Furthermore in injured or inflammed areas new barriers may be formed as exemplified

centa this localization being in all probability responsible for the cardinal clinical feature of the disease namely abortion (Smith *et al* 1961) The inability of antibodies and phagocytes to reach the fetal placenta cannot explain the unbridled proliferation of brucella organisms at this site for infection of pregnant cattle with various other types of bacteria does not show similar localization Extracts of fetal placenta infected or normal have been found to produce marked stimulation of the growth of brucellae but not of other microbes on artificial media or inside cells in tissue culture Extracts similarly prepared from various other tissues and organs of the same animals have no such action on brucella cultures The active agent in the fetal placenta extracts has now been isolated and identified as erythritol a 4-carbon polyalcohol (Pearce *et al* 1962) This substance is present in fetal placental tissues and fluids but not in various maternal organs or in the fetus itself (Williams *et al* 1962) The evidence thus suggests that trace amounts of this organic chemical which accumulate for reasons unknown in the fetal placenta determine by means of some vitaminlike effect on brucellae to a considerable extent the pathogenesis of infectious abortion in cattle Perhaps similar mechanisms at present totally unknown operate to determine localization of other bacterial infections at other organ or tissue sites

In addition to their action on invading microbes local physicochemical conditions in tissues also undoubtedly influence the function of the classic humoral and cellular host defense agencies In contrast with the situation in guinea pig tuberculosis mentioned above the kidney and especially the renal medulla exhibits striking susceptibility to sepsis in many other laboratory models of infectious diseases as well as in some clinical situations Detailed study of this phenomenon shows as the probable explanation for localization of disease a delayed and grossly deficient phagocytic reaction in the renal medulla (Rocha and Fekety 1964) The high salt concentration of interstitial fluids about the renal tubules might be responsible for impairing phagocytic function both by interfering with mechanisms of chemotaxis and with phagocytosis itself (Chernew and Braude 1962) Ordinary avirulent coliform bacteria also commonly produce infectious disease in the urinary tract Many of these microbes are killed by the combined action of antibody and complement and it has been suggested that the urinary tract manifests unusual susceptibility to sepsis by coliforms because the ammonia normally produced and excreted by the kidneys inactivates essential complement components thus impairing or eliminating completely this humoral defense mechanism (Beeson and Rowley 1959)

THE INFLAMMATORY REACTION

Thus far our discussion has dealt largely with interactions between bacteria and humoral cellular and tissue factors of the normal host In reality the conflict between host and microbe is waged not in normal body tissues or fluids but rather in a tissue environment modified by the conflict itself This modified tissue environment is termed inflammation or the inflammatory reaction (Florey 1962 Spector and Willoughby 1963)

When bacteria are introduced into tissues the irritating effects of the organisms themselves of toxins produced by them or of the procedure of their introduction set in motion a sequence of vascular humoral and cellular alterations The initial change in small blood vessels probably brought on by reflex nerve action is constriction Soon thereafter the vessels relax and enter into a stage of prolonged dilatation Blood flow through the dilated capillaries slows and the endothelial surfaces change so that leukocytes adhere to the vessel walls Vascular permeability increases due probably in the early phase of inflammation to the pharmacologic action of histamine and serotonin released from damaged mast cells in the area Plasma leaks out of the capillaries and the postcapillary venules and is diluted in the tissue juices leading to activation of enzymes and production of substances which alter the vessels so as further to increase their permeability Leukocytes adhering to the endothelium begin to move about and eventually crawl through the capillary wall probably

contact man via the oropharynx yet if disease results from this contact it typically appears in the pharynx with streptococci the lungs with pneumococci and the membranous coverings of the central nervous system with meningococci Many other examples could be given of organ or tissue localization of infectious agents which can not be attributed to the route of exposure This localization can hardly be based primarily on interactions of the bacteria with phagocytes or antibodies, since these are available to any part of the host via the bloodstream Local chemical and physical conditions must differ in various tissues and subtle features of this tissue microenvironment must determine whether or not a particular organism is able to survive and multiply

Biochemical conditions might determine the suitability of a given site for proliferation of bacteria in one of two ways A particular tissue in the host might contain antibacterial substances or conditions which kill or inhibit the microorganism or on the other hand nutrient or vitaminlike substances essential for virulent proliferation of the microbe might be lacking in some tissues but not in others This aspect of host resistance has been but little investigated and in most instances nothing is known about the local physiochemical conditions playing a role in determining the fate of invading bacteria Examples selected from among the few cases in which these microenvironmental agents have been studied will serve to emphasize and illustrate the phenomenon

Many chemical substances found in tissues do in fact exhibit antimicrobial activity under certain circumstances in vitro Long and short-chain fatty acids surface active lipids or proteins basic peptides amines and heme compounds occur in some tissues at concentrations higher than those which kill certain bacteria in the test tube Although studies along these lines are numerous in no instance is there convincing evidence of specific antibacterial action by one of these compounds in vivo (reviewed by Skarnes and Watson 1957 and by Hirsch 1960)

The difficulty of establishing the role of a tissue antibacterial substance in determining the course of an infectious process may be illustrated by the following example The guinea pig is notoriously susceptible to tuberculosis yet the guinea pig kidney exhibits remarkable resistance to this same disease If virulent tubercle bacilli are inoculated directly into the guinea pig kidney the animal develops a progressive fatal illness At autopsy, extensive tuberculosis is found in many organs and tissues but the kidneys regularly appear to be spared even the original renal inoculation site is healed completely (Birkhauser, 1950) With this phenomenon in mind studies have been done attempting to extract from kidney tissue a substance capable of killing or inhibiting tubercle bacilli in culture A crystalline compound with potent antimycobacterial activity has been isolated from the kidney extracts and identified as spermine (Hirsch and Dubos 1952) an organic amine of unknown function distributed widely and variously in tissues Further study in the laboratory on the mechanism of action of spermine on tubercle bacilli has revealed that spermine is in fact not itself directly inhibitory for the organisms rather spermine is degraded by an amine oxidase present in the culture medium and it is some product of this enzymatic attack which then acts to kill or block growth of the microbes (Hirsch 1953) Thu possible antimycobacterial activity of spermine in tissues if it occurs would depend not only on the tissue concentration of spermine but also on the concentration of the amine oxidase and on the presence of physicochemical conditions conducive to the enzymatic reaction and stabilization of the antibacterial product The complexity of this situation has made it impossible to reach definitive conclusions on the role of this system in determining the fate of tubercle bacilli in the infected animal

An example of a tissue chemical agent which favors growth of a specific microbe and may determine the localization of an infectious disease comes from very recent work on the pathogenesis of brucellosis Study of the distribution of viable *Brucella abortus* in various organs and tissues of infected cattle when abortion is imminent shows a striking pattern of the 10^{14} brucellae found in the entire animal approximately 75 per cent are localized in the fetal pla

in most instances understood poorly or not at all but they might act directly or indirectly (1) to alter capacity of the microbe for virulent behavior (2) to affect one or more of the basic host resistance mechanisms—anatomic barriers phagocytic cells humoral factors or local tissue biochemistry or (3) to change the timing or the qualitative or quantitative nature of the inflammatory response

Obviously no attempt can be made here to review thoroughly the broad range of environmental effects and organic disorders which alter more or less resistance to infectious agents and their toxins Rather we shall discuss briefly a few examples to illustrate the wide range of factors which come into play and to point out the general lack of understanding of mechanisms in this field

Hormones and Host Resistance

Hormones secreted by the various endocrine glands serve to regulate a wide variety of physiologic functions Circumstantial and in some cases direct evidence indicates that many of these hormones exert an influence on host resistance mechanisms (reviewed by Kass 1960)

Many observers have commented on relationships between thyroid function and susceptibility to infections The reports are somewhat conflicting with the effects of too much or too little thyroid hormone apparently varying depending on the host and the parasite species studied In experimental tuberculosis in rabbits hyperthyroidism is associated with increased host resistance whereas hypothyroid animals are more susceptible than normal and develop spreading disease (Lurie *et al* 1959a b) On the other hand hyperthyroidism results in lowering of resistance of rats and mice to experimental streptococcal or staphylococcal infections (Reichlin and Glaser 1958 Smith and Dubos 1956)

Epinephrine administered locally say into the skin can interfere profoundly with the host s ability to handle various bacteria injected into the same site (Miles *et al* 1957) The primary action of the hormone in this situation appears to be a protracted vasoconstriction with as a result delay in the development of an inflammatory response and alteration in the tissue microenvironment

Most extensively studied of the hormonal effects on infectious disease is that of the adrenal glucocorticoid hormones These hormones have been used widely during the past decade for treating various diseases Their administration in large doses to man or various animals is regularly accompanied by an impressive increase in susceptibility to invasion by a wide variety of microorganisms including ordinarily avirulent species or latent microbes which are somehow awakened from their state of hibernation to become invasive There is no evidence that cortisone exerts any direct effect on microbes rather it somehow produces alterations in basic host defense mechanisms (reviewed by Germuth 1956 Robinson 1956) An animal under the influence of excess adrenal glucocorticoids exhibits striking suppression of the inflammatory response to a wide variety of noxious agents including invading microbes Cortisone apparently somehow prevents the increased capillary permeability and the leukocyte emigration which underlie inflammation this action undoubtedly accounts at least in part for the lowered host resistance to infection Cortisone and similar steroids also influence carbohydrate and protein metabolism and thus possibly alter the local tissue biochemical environment so as to render it more suitable for microbial proliferation no definitive evidence on this possibility is available Very high levels of cortisone can lead to suppression of antibody formation but this action is probably not primarily responsible for the depressed host resistance (Germuth 1956) Functional capacity of polymorphonuclear leukocytes and of reticuloendothelial macrophages appears not to be significantly altered by excess adrenal hormones although there is some disagreement on this point (see Germuth 1956 Hirsch and Church 1961)

The Influence of Various Diseases on Host Resistance

Various disseminated diseases especially in their terminal phases are associated with a striking increase in susceptibility to infectious agents Patients with leukemia carcinomatosis uremia and other severe illnesses

by penetrating between endothelial cells Through a poorly understood mechanism of attraction called chemotaxis, the leukocytes move toward the invading organisms and if conditions are suitable engulf and destroy them Excessive exudation of fluid into the inflamed area leads to dilatation of lymphatic vessels and increased drainage into regional lymph nodes Microbes caught up in this lymph drainage are filtered in the nodes, being taken up by fixed mononuclear phagocytes which are part of the reticuloendothelial system Fibrinogen in the extravasated fluid may clot particularly at the periphery of the inflamed zone to form a fibrin barrier serving to limit direct spread of the microbes and influencing fluid and cellular exchange between the inflammatory area and the surrounding normal tissue

Thus when bacteria gain access to tissues a race of sorts ensues The outcome depends in no small measure on the rapidity and the nature of the inflammatory reaction which provides a mechanism for the controlled escape of humoral and cellular resistance agencies from the bloodstream so that they may establish contact with the invading microbes

The physicochemical features of the inflammatory site remain to be studied thoroughly but they differ in many regards from those of normal tissue Blood stasis, influx of phagocytic cells with glycolytic metabolism growth of the microbes themselves, damage and even death of host cells produce in the inflamed zone a slightly acid pH a lowered oxygen tension retention of CO accumulation of lactic acid and undoubtedly numerous other changes (Dubos 1954 1955) Some of these biochemical alterations may act, for instance by damaging blood vessels to perpetuate or increase the inflammatory reaction they also undoubtedly affect both the capacity of the microbes to thrive and the functional efficiency of classic humoral and cellular defense systems

An inflammatory reaction which is overly vigorous or uncontrolled multiplication of the bacteria may lead to tissue necrosis with further changes in the microenvironment in which the battle is fought Of course tissue necrosis is not the result to be desired by the host but in at least some situations necrosis and the attendant local biochemical changes may serve to limit further multiplication of the parasite and act ultimately as a host defense mechanism Consider for instance microscopic features of the typical lesion of tuberculosis the tubercle (Canetti 1955) The center of such a lesion is composed of structureless partially autolysed necrotic tissue, and surrounding this central core is a cuff of viable tissue heavily vascularized and infiltrated with phagocytic cells Tubercle bacilli die off and disappear from the central necrotic area whereas they continue to thrive in the surrounding inflamed living tissue Thus is presented the paradoxical situation of continued growth of the invading microbes in the area containing an abundant supply of the classic host defense systems humoral and cellular, and death of the organisms in the avascular, acellular zone[1]

So little is known about the chemical composition of necrotic tissue that no definitive statements are possible as to the physicochemical components which might exert antimicrobial effects therein Accumulation of fatty acids porphyrins and amines liberation of basic proteins and peptides and release of antibacterial substances from disrupted phagocytic cells may well be responsible at least in part for suppressing microbial growth in such areas (see Hirsch 1960) Synergists and antagonists for these antibacterial substances from tissue are probably always present or readily available in an infected area and no doubt determine in no small measure whether or not in vivo activity can occur Our knowledge of the subtle and dynamic biochemical aspects of the local site of the host parasite encounter is primitive indeed yet it is likely that just these aspects determine directly or indirectly the fate of the invading microbes

FACTORS THAT ALTER HOST RESISTANCE TO INFECTION

Many disease processes or environmental factors result in disturbances of general host physiology which are associated with altered resistance to infectious diseases The precise means by which these abnormal physiologic states influence the host parasite conflict are

of the intestinal flora (Dubos and Schaedler 1962) Even severe degrees of malnutrition do not significantly impair antibody formation or grossly interfere with phagocyte performance or the inflammatory reaction

The widespread notion that better than average nutrition leads to better than average resistance to infections appears to be a misconception There is little evidence that supplementation of ordinary diets with vitamins or other factors has any effect on host resistance (see Scrimshaw *et al* 1959)

In fact in a few instances dietary deficiencies seem to be associated with increased ability to ward off microbial invaders An example of such a phenomenon is the resistance to malaria of rats or monkeys on a milk diet When this association was first noted it was assumed that milk might contain some resistance factor for malaria Further study has shown that the milk diet is deficient in a vitaminlike substance para aminobenzoic acid required for growth of malaria parasites Resistance while on the milk diet is due to this deficiency for supplementation with the acid promptly renders the animals susceptible to malaria infections (Hawking 1954)

The Influence of Weather on Infectious Diseases

Most infectious diseases display a rather regular pattern of seasonal incidence—streptococcal pharyngitis is most common in late winter or early spring viral influenza occurs most frequently in early winter infantile diarrhea due to coliform organisms prevails in the summer etc These seasonal patterns are commonly explained by prevalence of insect vectors by variations in sanitary conditions by crowding of the host or by effects of temperature humidity and sunlight on survival of the microbe outside the host However for many infectious diseases seasonal patterns are completely unexplained

Such seasonal fluctuations may also be due at least in part to effects of the weather on host physiology and resistance mechanisms as is indicated by the following example In the course of studies on streptococcal infection of mice in an aerosol chamber Coburn and associates (1957) noted that the animals developed severe often fatal diseases when the experiments were done in winter months whereas during the summer the mice showed no illness following similar exposure To study the phenomenon further a large batch of virulent streptococci was cultured and then divided into small lots which were preserved by freeze drying so that essentially identical bacteria could be used for the inoculations over the course of 2 or more years Then mice were exposed to the streptococcal aerosol under standard conditions every month Cultures taken from the animals after exposure established that similar numbers of viable bacteria were introduced in all seasons thus contact between mice and the streptococcal bacteria was the same Yet over the period of observation the mice so exposed developed streptococcal infectious disease often fatal only during winter and spring months In the summer and the fall no disease resulted These results thus indicate that in this particular model season or weather may influence the occurrence of infectious disease by affecting the physiology of the host somehow to alter suceptibility to sepsis

Similarly recent studies on experimental transmission of viral influenza in mice also suggest that seasonal factors may somehow influence host resistance (Schulman and Kilbourne 1963)

Many different types of environmental factors are included under the terms weather or season and few studies have been done attempting to relate isolated meteorologic factors and susceptibility to infectious diseases Temperature may well play a role in this regard for animals exposed to cold exhibit impaired resistance to salmonellosis and staphylococcal infections (Miraglia and Berry 1962)

Role of the Normal Flora in Host Resistance

Host resistance to sepsis is influenced by the nature of the normal microbial flora As stated earlier skin mucous membranes of the upper respiratory tract and intestines are swarming with myriads of microbes living in peaceful coexistence with the host When this normal flora is eliminated or altered drastically by germ free technic or by administration of antimicrobial drugs striking

often develop sepsis the invading microbe may be one of the avirulent members of the indigenous flora Information is not available on the precise alterations in host resistance mechanisms in this setting

Persons afflicted with diabetes mellitus are unusually prone to develop cellulitis or boils and seem to exhibit lowered resistance to infectious diseases in general The resistance to experimental fungal infections of animals made diabetic by administration of alloxan is depressed only if their diabetes is uncon trolled to the extent that acidosis develops Detailed investigation of the host parasite interaction in these diabetic and acidotic animals shows as a possible basis for their increased susceptibility a delayed and grossly deficient influx of phagocytic cells into the challenge site (Sheldon and Bauer 1959) Metabolic derangements in diabetes doubtless also lead to changes in local tissue chemistry which may well influence the course of incipient infectious disease

Disturbance of host resistance is by no means limited to generalized or severe dis eases Local abnormalities sometimes exert a profound effect on pathogenesis of infec tions As discussed in an earlier section an endocardial scar resulting from an attack of rheumatic fever often becomes the site of local invasion by usually benign microbes with resulting production of the serious disease subacute bacterial endocarditis Other local abnormalities also can result in ineffective host resistance For example ob struction to the flow of any drainage system —urinary tract respiratory tree bile canals etc —is almost uniformly associated with invasion of the obstructed system by bacteria of the normal flora and production of severe and often persistent infectious disease The precise mechanisms which account for local paralysis of host defense in this setting are not clearly established altered local physical and chemical conditions which result from obstruction probably affect both the para site and the host response

Disease caused by one microbial agent may also influence host resistance to another unrelated microorganism The striking in crease in susceptibility to bacterial pneu monia which follows an attack of viral influenza exemplifies this phenomenon Mechanisms underlying this relationship are not well understood

Host resistance may also be altered strik ingly by the therapy administered for other diseases As mentioned above multiple host resistance agencies are affected by cortisone and related steroids Increasing use is now being made of certain antimetabolites and alkylating drugs for cancer chemotherapy and for suppression of transplantation immunity these drugs often impair both cellular and humoral resistance mechanisms and patients who receive them show unusual susceptibility to infectious diseases Similar or even more marked impairment of resistance to sepsis is seen in persons or animals exposed to large doses of gamma or x irradiation which result in breakdown in anatomic barriers cessation of phagocyte production and paralysis of antibody formation (Miller 1956)

Nutrition and Infection

Long before the modern microbial era it was recognized that a relationship existed between famine and epidemic contagious disease History continues to record this relationship during World War II striking outbreaks of infectious disease occurred among prisoners-of war deprived of adequate food

The literature on relationships between nutrition and infection is voluminous and disconcertingly inconclusive (reviewed by Schneider 1946 Scrimshaw et al 1959, Dubos and Schaedler 1959) In some in stances the diet has important effects on the invading microbe and on the mechanisms of the host response however these effects often vary depending on the particular microbe and host and are intimately inter woven with other aspects of host physiology

Even a relatively short period of starva tion renders experimental animals unusually susceptible to microbial challenge Imbalance in the diet and especially shortage of ade quate protein nutrients can lead to similar effects (see Dubos and Schaedler 1959) Little is known about the precise chain of events involved in depression of resistance by these nutritional disturbances The effect may be a direct one or in some instances it may operate through associated disturb ances e g hormonal changes or alteration

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changes take place in susceptibility to certain infectious diseases For example bacillary dysentery cannot be produced in ordinary guinea pigs even when large doses of virulent shigella organisms are given However ad ministration of the same microbes to germ free guinea pigs gives rise to fatal ulcerative enteritis (Sprintz *et al*, 1961) The increased incidence of staphylococcal entero colitis and of moniliasis during the course of long term therapy of persons with broad spectrum antibiotics is an example of the same phenomenon from the field of clinical medicine

Strictly speaking the normal flora cannot be classified as a host resistance mechanism since the microbes are outsiders living with but not part of the host Their role in deter mining pathogenesis of infectious diseases is nevertheless important How does the normal floral protect the host from potentially harm ful bacteria?

The most logical explanation would in voke a kind of interference As an analogy a dense healthy stand of good grass is relatively resistant to growth of airborne weed seeds alighting upon it whereas a barren field provides a fertile area for un controlled growth of weeds The skin or the mucosal surfaces of mammals perhaps pro vide sufficient nutrients for survival of only a finite concentration of microorganisms If the normal flora effectively utilizes this supply of nutrients invaders find it difficult or im possible to establish a foothold Or an alter native explanation would involve production by the normal flora of substances (antibi otics) or conditions (acidity low oxygen tension etc) inhibitory for foreign microbes attempting to establish residence in the area

Some evidence suggests furthermore that the normal flora affects resistance of the host to infection at sites far removed from the habitat of the flora itself Thus it is also possible and even likely that the normal flora exerts a significant influence on general host physiology, perhaps by providing vita mins, by exposing the host continuously to small doses of endotoxins or by affecting directly or indirectly *antibody producing* systems or turnover of phagocytic cells These physiologic effects might well be re flected in alterations in the ability of the host to handle a given challenge of infectious material

CONCLUSION

Pathogenesis of infectious diseases seems at first glance to be relatively simple and well understood The environment in which man lives is swarming with microbes of all sorts some of these are capable of virulent be havior based on specific structures which they possess or poisons which they produce To ward off the challenge of these potentially virulent bacteria man calls into play his defense mechanisms anatomic barriers phagocytic cells and antibodies

Our purpose in this chapter has been to examine anew the mechanisms of resistance to infectious disorders in the light of modern knowledge and concepts emphasizing the unknown as well as the known These mech anisms are multiple are in many instances still understood poorly or not at all and probably differ from microbe to microbe from person to person and from time to time Host resistance is determined not only by antibodies and phagocytes but also by a myriad of subtle factors some local some general and some entirely separate from the host itself

The pathogenesis of infectious diseases in many ways illustrates well the infinite complexity of biologic systems with living things existing in a state of delicate balance under the constant influence of interactions between themselves and their environment

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9

The Role of Antibody, Complement and Other Humoral Factors in Host Resistance to Infections

INTRODUCTION

Host defense to infectious agents is a complex biologic process requiring participation of cells as well as humoral factors In this chapter we shall deal exclusively with the nature and the function of humoral factors It is to be emphasized that separate treatment of the humoral aspect represents an abstraction from the actual biologic phenomenon in the living organisms cellular and humoral defense mechanisms are interdependent and intimately interwoven

Serum factors contribute to host defense essentially in three ways neutralization of bacterial toxins and viruses destruction of microorganisms and promotion of phagocytosis The factors primarily responsible for these effects are antibody complement and opsonin Initially these terms designated merely serologic activities Today at least some of these activities can be ascribed to discrete serum protein entities which are now being subjected to thorough physical and chemical analysis The achievements of recent years encourage the belief that it will be possible in the foreseeable future to describe the humoral mechanisms of host defense entirely in terms of the underlying molecular events Wherever feasible the molecular aspect will be preferred to the serologic throughout the following discussion

HISTORY

The chemical approach to problems of immunity was introduced by Paul Ehrlich He believed so firmly in the chemical mode of action of antibody and complement that he was led to attempt imitation of their biologic activity with the aid of compounds synthesized in the laboratory When Ehrlich began his work on the mechanism of immunity the cellular theory of immunity was well established Metchnikoff had drawn wide attention to the crucial role of the migrating phagocytic cell in host defense against invading microorganisms He regarded the phagocytic cell as solely responsible for the phenomenon of natural immunity and as the primary force in acquired immunity Nutall s demonstration of bactericidal power of serum was attributed by Metchnikoff to an artefact He ascribed it to the release of bactericidal substances from leukocytes on their disintegration in vitro That phagocytosis required the presence of serum was in his opinion due to the sustaining effect of serum on cell function (Metchnikoff 1905)

During the latter part of the last century theories of biology and medicine were based on cellular phenomena Humor was a notion which was colored and burdened with the memory of obsolete speculations Therefore theories invoking the body fluids met with

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Serum factors contribute to host defense essentially in three ways neutralization of bacterial toxins and viruses destruction of microorganisms and promotion of phagocytosis The factors primarily responsible for these effects are antibody complement and opsonin Initially these terms designated merely serologic activities Today at least some of these activities can be ascribed to discrete serum protein entities which are now being subjected to thorough physical and chemical analysis The achievements of recent years encourage the belief that it will be possible in the foreseeable future to describe the humoral mechanisms of host defense entirely in terms of the underlying molecular events Wherever feasible the molecular aspect will be preferred to the serologic throughout the following discussion

HISTORY

The chemical approach to problems of immunity was introduced by Paul Ehrlich He believed so firmly in the chemical mode of action of antibody and complement that he was led to attempt imitation of their biologic activity with the aid of compounds synthesized in the laboratory When Ehrlich began his work on the mechanism of immunity the cellular theory of immunity was well established Metchnikoff had drawn wide attention to the crucial role of the migrating phagocytic cell in host defense against invading microorganisms He regarded the phagocytic cell as solely responsible for the phenomenon of natural immunity and as the primary force in acquired immunity Nutall s demonstration of bactericidal power of serum was attributed by Metchnikoff to an artefact He ascribed it to the release of bactericidal substances from leukocytes on their disintegration in vitro That phagocytosis required the presence of serum was in his opinion due to the sustaining effect of serum on cell function (Metchnikoff 1905)

During the latter part of the last century theories of biology and medicine were based on cellular phenomena Humor was a notion which was colored and burdened with the memory of obsolete speculations Therefore theories invoking the body fluids met with

suspicion While cells and the phagocytic process could be comprehended easily by *microscopic observation* there was no microscope at hand to visualize serum constituents and to facilitate the understanding of their function Nevertheless the basic concepts of humoral immunology were evolved then and they have retained their validity to the present Due to a complete lack of information regarding the composition of serum the terminology created by the early students of antibody and complement is often romantic and awkward and tends to obscure the extent of their contribution

Soon after Nutall s discovery of the bactericidal power of serum Pfeiffer (1903) found *bacteriolytic activity* to be increased in the serum of animals that had survived a bacterial infection The serum would lyse only those bacteria with which the animal had been infected The activity could be transferred with cell free serum to a non immune recipient It was thermostable and this property distinguished it from the heat labile bactericidal principle which Buchner attributed to an enzyme in serum Thus there appeared to be two humoral principles and it was soon recognized that both were required for bacteriolysis The heat stable factor with specificity for microorganisms was later designated as antibody and the heat labile enzymelike substance as complement It was Ehrlich (1906) who formulated the final concept of immune cytolysis according to which antibody combines by means of a specific combining site with a receptor on the cell surface Complement secondarily attaches itself to a receptor site on the antibody molecule and thereby is enabled to execute its damaging effect on the cell membrane

Bordet s discovery of immune hemolysis greatly facilitated the study of serum factors with cytolytic activity It provided a simpler experimental system and a means for precise quantitation of the effect of antibody and complement More importantly it led to the realization that immune cytolysis is not confined to microorganisms but concerns cells in general thus forming the basis for the concept developed later that antibody and complement not only participate in host defense against pathogenic microbes but also against heterologous and isologous cells transferred from another organism This view now has been broadened even further to include the possibility that complement might be capable of damaging autologous cells in diseases with an immunologic basis

The key idea in the young field of humoral immunology was that of the specificity of antibodies In 1890 Emil von Behring discovered that animals which had been infected with diphtheria produced antibody to diphtheria toxin The antibody was able to confer immunity on another animal which had not been previously exposed to either the microorganism or the toxin and the protective effect was entirely specific Almost simultaneously Ehrlich carried out experiments with the plant poisons ricin and abrin and demonstrated that an antiricin antibody could neutralize the effect of ricin but not of abrin and vice versa In defining *antibody specificity* Ehrlich resorted to the principles of enzymology Emil Fischer had compared the fit between enzyme and substrate with the relationship between key and lock It was this highly specific relationship that Ehrlich visualized to exist between antibody and antigen The subsequent work of Landsteiner and that of Avery and his collaborators revealed how sensitive a tool an antibody is for the detection of minute differences in the structure of antigenic determinants

The earliest phase of chemical immunology began when it became possible to study antigen antibody reactions in vitro Since the plant poisons ricin and abrin have the ability to agglutinate the red cells of certain species inhibition of agglutination could be used as a measure of *neutralization of toxin* by antitoxin Using this method Ehrlich established a quantitative relation between the dose of antitoxin and the degree of neutralization He also demonstrated with these experiments that the neutralization of toxin is a purely humoral process which does not require cells

The term neutralization was chosen by Ehrlich because he firmly believed that the interaction between antigen and antibody was basically similar to an acid base reaction Arrhenius adopted this view and developed it by applying the law of mass action to the theoretical treatment of immune complexes Like other chemical reactions the union of

antigen and antibody was considered to be reversible and the equilibrium dependent on the concentrations of the reactants and the reaction product Arrhenius thus introduced methods and principles of physical chemistry into immunology Incidentally he was the first to speak of the science of immuno chemistry

After the basic concepts of chemical immunology had been developed during the latter part of the last century interest turned to the elucidation of the nature of antibody and complement and of the structure of anti genic determinants This new period of activity began in the mid twenties and is still continuing

ANTIBODY

Antibodies are serum proteins which are uniquely endowed with an affinity for a mul titude of naturally occurring and synthetic substances called antigens Each antibody molecule carries specificity for only one anti genic group In general antibody molecules possess two sites capable of combining with antigen By virtue of their combining sites antibodies can give rise to the formation of soluble antigen antibody complexes insol uble immune precipitates and to aggluti na tion of cells and particles if antigen is bound to their surface Antibodies themselves anti genic belong to the immunologically defined family of γ globulins

Localization of Antibody Activity in γ Globulins

The proteins of serum may be separated in an electric field into 4 major fractions of different electrochemical properties In the order of their mobility at pH 8 6 they are designated albumin α β and γ globulin the last being the slowest migrating fraction To determine the relation of the eletropho retic fractions to antibody activity of serum Tiselius and Kabat (1939) compared the patterns of normal sera with those of hy perimmune sera Hyperimmune sera differed strikingly from normal controls in that the γ globulin fraction was conspicuously in creased On absorption with homologous antigen the γ globulin concentration in im mune sera was reduced to a normal level With these observations Tiselius and Kabat established that antibody activity is a function of the γ globulin molecule Today the terms antibody protein and γ globulin are often used synonymously and it is thought prob able that all γ globulin occurring in normal serum represents specific antibody This view is strengthened by observations of animals raised in a germ free environment γ globulin levels in such animals are often as low as 10 per cent of the normal and they rise rapidly on exposure of the animals to an ordinary environment (Gustafsson and Laurell 1959)

Since the early days of electrophoretic investigation when γ globulin was defined by the physical chemist as the slowest migrating fraction of serum the story of γ globulin has become immensely complex This devel opment began when immunochemists dis covered that small amounts of protein closely related to γ globulin are found with great regularity in the β and even the α globulin region of the electrophoretic spectrum (Slater 1955 Williams and Grabar 1955) Today therefore an immunochemical defini tion is preferred according to which all proteins that are recognized by antibody to classic γ globulin are members of the γ globulin family irrespective of electropho retic mobility This group of related proteins is comprised of 7S γ globulin 19S γ globulin and β_A globulin including their pathologic counterparts the myeloma proteins and the macroglobulins of the Waldenstrom type The low molecular weight γ_L globulin of serum and normal and pathologic urine also belongs to this group With identification of the β proteins and the 19S proteins as γ globulins the question arose whether these proteins also have antibody activity

There is no doubt that 19S γ globulin possesses antibody activity In fact, the earliest studies of hydrodynamic aspects of purified antibodies disclosed the occurrence of active 19S components in hyperimmune sera (Heidelberger and Pedersen 1937 Kabat 1939) Later certain antibodies of human origin such as the Wassermann anti body and the antibody to the somatic O anti gen of the typhoid bacillus were found to be of high molecular weight (Davis *et al* 1945 Deutsch *et al* 1946) In recent years Kun

TABLE 1 PROPERTIES OF THE HUMAN GAMMA GLOBULINS

	7S γ GLOBULIN	19S γ GLOBULIN	β_{2A} GLOBULIN	γ_L GLOBULIN
Approximate serum concentration	1 000 mg %	50 100 mg %	60-200 mg %	trace
Sedimentation coefficient	7S	19S	7 15S	1S
Molecular weight	160 000	1 000 000	150 000 600 000	25 000
Carbohydrate content	2 6%	10 5%	10%	0%
Antibody activity	present	present	present	absent
Pathologic counterpart	γ Myeloma protein	Waldenstrom s macroglobulin	β_{2A} Myeloma protein	Bence Jones protein

kel (1960) has studied a larger series of human antibodies and has demonstrated conclusively the existence of an entire class of antibodies of high molecular weight To it belong certain isoagglutinins and antibodies against the M the N and the P antigens of human red cells normal and pathologic cold agglutinins and heterophile antibodies as well as certain autoantibodies occurring in patients with lupus erythematosus and similar diseases Rheumatoid factors are also 19S antibodies (Kunkel *et al*, 1959) they are of particular interest since they exhibit a high degree of specificity for autologous and isologous γ globulin and have permitted the discovery of hereditary γ globulin groups

Much more difficult to answer has been the question whether β_{2A} globulin contains antibody activity Some evidence suggests the occurrence of antibodies against bacteria and viruses (Schultze 1959) and of reaginic activity (Heremans *et al* 1962) in this fraction There has also been the suggestion that a new ultracentrifugal species of antibodies (9 15S) might be related to β_A globulin primarily because their sedimentation behavior resembles that of β_A polymers (Rockey and Kunkel 1962) Using highly specific antiserum to β_A globulin Fireman *et al* (1963) succeeded in absorbing β_{2A} globulin from human serum virtually without affecting the concentration of the other two γ globulins When this procedure was applied to sera containing skin sensitizing antibodies to ragweed pollen the reaginic activity was eliminated completely together with β_A-globulin These elegant experiments established conclusively that antibody activity does reside in this fraction Thus all 3 major γ globulins have been shown to have antibody function

TYPES OF γ GLOBULIN AND THEIR PROPERTIES

There are 4 principle types of γ globulin which are related to each other through common antigenic determinants In terms of molecular structure the 4 γ globulins share certain polypeptide chains but differ with respect to others

7S γ Globulin represents approximately 10 per cent of total serum protein Its molecular weight is in the vicinity of 160 000 The predominant N terminal amino acids are aspartic and glutamic acid A carbohydrate moiety accounts for 2 6 per cent of the weight of the molecule and is composed of galactose mannose fucose hexosamine and neuraminic acid While 7S γ globulin appears to be quite homogeneous when examined in the ultracentrifuge it exhibits a perplexing heterogeneity on electrophoresis The electrophoretic mobility spectrum ranges from -0.5 to -3×10^{-5} cm² sec⁻¹ V⁻¹ Approximately 80 per cent of the protein behaves electrophoretically as classic γ globulin The rest has mobility values of β and α globulins (Slater 1955 Williams and Grabar, 1955) This fact was first revealed with the aid of the immunoelectrophoretic method and prompted definition of γ globulin in terms of antigenic rather than electrochemical properties

The wide electrophoretic distribution of 7S γ globulin is due to the presence in this fraction of a large number of discrete molecular species each presumably derived from an individual clone of plasma cells That individual plasma cell clones are able to produce γ globulins with an extremely narrow mobility distribution is apparent from the behavior of myeloma proteins and of certain

antibodies (Kunkel et al 1963) They may give rise to unusually sharp protein peaks Originally the sharpness of their peaks as well as some other features were believed to be reflections of an abnormal protein structure but present evidence suggests that myeloma proteins are only abnormal elevations of otherwise normal individual species of γ-globulin In many recent studies therefore they have been utilized to obtain information on monoclonal γ globulins which for technical reasons could not be obtained on normal serum As will be seen below myeloma proteins and highly purified antibodies resemble each other in certain structural features Therefore it is tempting to relate the molecular heterogeneity of 7S γ globulin to the multitude of different antibody specificities which reside in this protein Different specificities require differences in combining regions which in turn may produce dissimilarities in over all molecular characteristics On the other hand molecular heterogeneity may be an expression of complexity of the cell population from which the protein originated

19S γ Globulin This protein was not clearly recognized as a separate entity until it was isolated and shown to be chemically different from 7S γ globulin (Muller Eberhard *et al* 1956) It contains a carbohydrate moiety which accounts for more than 10 per cent of the molecule and is 4 times larger than that of 7S γ globulin The individual carbohydrate constituents are the same as in 7S γ globulin but their relative proportions differ The protein has a molecular weight of 1 million and an electrophoretic mobility intermediate to γ and β globulin

The most interesting feature of this protein is that it is composed of a number of subunits probably 6 which are held together by disulfide bonds By treating 19S γ globulin with disulfide bond-cleaving reagents Deutsch and Morton (1957) were able to dissociate it into 7S subunits With this discovery they called attention to the significance of S S bonds for the structure of γ globulin and stimulated studies concerned with the chemical dissociation of γ globulin into polypeptide chains The role of intramolecular S S bonds in the activity of 19S antibodies is revealed by the effect of reduction and reoxidation Reduction with mercaptoethanol is accompanied by complete loss of activity (Fudenberg and Kunkel 1957) subsequent reoxidation leads to reactivation (Kunkel *et al* 1961)

The pathologic counterparts of 19S γ globulin are the macroglobulins of the Waldenstrom type If the pathologic γ globulins represent unusually elevated normal proteins then it ought to be possible to find antibody activity associated with at least some of them While such observations have not been reported for myeloma proteins with one possible exception (Waldenstrom 1961) antibody activities have definitely been found for some of the Waldenstrom macroglobulins (MacKay 1959 Kritzman *et al* 1961)

β_{A} Globulin This serum component was discovered in immunoelectrophoretic studies by Grabar and Williams (1953) It migrates in the region between γ and β-globulin The carbohydrate content of the purified material is approximately 10 per cent (Heremans 1960) It occurs in several molecular species the major component has an s rate of 7S the minor components of 9 15S The faster sedimenting material can be dissociated into 7S units by reduction with mercaptoethanol (Fahey 1961)

γ_L Globulin To the 3 categories of γ globulins discussed so far a 4th one has been added recently Evidence for its existence was first obtained when a γ globulin of low molecular weight was isolated from normal human urine (Webb *et al* 1958 Franklin 1959) Originally it was thought to be a breakdown product derived from normal serum γ globulin but now it is considered to arise from *de novo* synthesis Interest in γ_L globulin was stimulated considerably when Berggård (1961) demonstrated its occurrence in normal human plasma Its concentration in plasma is so low that it can be recognized only after separation from the bulk of the serum proteins by ultrafiltration and subsequent concentration The best estimates indicate a molecular weight of 25 000 which is surprisingly low for a serum protein therefore it was named γ_L globulin (L stands for low molecular weight) Th immunologic identity of γ_L of plasma and of urine has been established (Berggård 1961)

γ Globulins of low molecular weight are known to occur in the urine of patients with

multiple myeloma. Such patients excrete large amounts of the so-called Bence Jones protein which is also characterized by peculiar thermosolubility properties Bence-Jones proteins precipitate at temperatures between 50 and 60 C and become soluble again at higher temperatures These proteins have been identified with a well characterized species of polypeptide chains the so-called L chains, of normal and pathologic γ globulins (Edelman and Gally 1962) Since γ_L globulin was found to resemble L chains in several important respects (Berggard and Edelman, 1963), it is now regarded as the normal counterpart of Bence Jones proteins

Present evidence indicates that the isolated light polypeptide chains of antibodies do not have antibody activity (see below) Inasmuch as γ_L-globulin consists of light chains antibody activity would not be expected to reside therein Contrary to this reasoning one group of investigators (Remington *et al* 1962) has reported poliovirus neutralizing activity and specificity to tetanus toxin to be present in urinary γ_L globulin of an immunized person

Genetic Types of Human γ Globulin

The existence of genetic types of γ globulin was discovered by Grubb and Laurell (1956) To date 7 genetic factors are known The 4 major ones are called Gm (a) Gm(b) Inv(a) and Inv(b) They are determined by genes present at 2 loci the Gm and the Inv The genetic properties are detected by a method utilizing rheumatoid factors which have anti Gm and anti Inv specificity Gm(a) and Gm(b) sites are found only in 7S γ globulin They are absent from 19S γ globulin β_A globulin and Bence Jones proteins By contrast the Inv character is a property of all 4 γ globulins It follows from the distribution of the genetic sites that the Inv character must be located on a type of polypeptide chain which occurs in all 4 proteins whereas the Gm site should be located in a region of the molecule which is peculiar to 7S γ globulin Indeed it has been possible to localize the Inv and the Gm properties to different parts of the 7S γ globulin molecule The Inv property is located in the so-called S-fragment and the Gm site in the F fragment (Harboe *et al* 1962 Franklin *et al* 1962) Only the F fragment is unique to 7S γ globulin whereas the S fragment contains groups that are common to the entire γ globulin family

Structure of γ Globulin

Elucidation of the structure of a protein with a molecular weight of 160 000 is a formidable task In the case of γ globulin this task is particularly difficult owing to the existence of a bewildering microheterogeneity within the protein Isolation from normal serum of homogeneous species of γ globulin or of antibody directed to a single antigenic determinant is not possible at present In spite of these difficulties the problem of γ globulin structure is being pursued actively in a number of laboratories Interest centers on the question of what constitutes the chemical basis of antibody specificity Is there a region in the molecule with a high degree of initial flexibility which is potentially capable of assuming many different conformations? Or is there a segment which varies in amino acid composition from one antibody to another? In short is specificity determined by primary or by secondary and tertiary structure? In pursuing these questions several groups of investigators recently have succeeded in elucidating some general structural features of the γ globulin molecule The over all objective has been separation of the molecule into structural and functional subunits Two methods have been used hydrolysis with proteolytic enzymes and chemical dissociation

Fragmentation by Enzymatic Hydrolysis In 1958 Porter separated papain-digested rabbit antibody on carboxymethyl cellulose and obtained 3 distinct products Further work (Porter 1959 Nisonoff *et al* 1960 Palmer *et al*, 1962) yielded the following information Treatment of rabbit 7S antibody with papain in the presence of cysteine produces 3 fragments called I II and III Fragments I and II have a molecular weight of 50,000 They are highly similar or even identical with each other for a given antibody molecule and cannot be separated Fragment III has a molecular weight of 80 000 and can be crystallized It differs from the other 2 pieces in many respects Piece I and piece II each contain one antibody-combining site

Being univalent they are unable to precipitate homologous antigen Yet they are capable of inhibiting specific precipitation by intact antibody Piece III is entirely devoid of specific antibody activity but it is responsible for placental transfer of the intact 7S molecule and for its ability to fix to skin which is a prerequisite for cutaneous anaphylaxis (Ovary *et al* 1961) In the undegraded molecule pieces I and II are linked to each other by 1 rather labile disulfide bond and both are linked to piece III by peptide bonds If pepsin is used for degradation instead of papain activated by cysteine the labile disulfide bond is not split A bivalent 5S antibody results which consists of 2 univalent pieces held together by 1 disulfide bridge Fragment III becomes dialyzable under these conditions

Enzymatic degradation has also been applied to the study of the antigenic structure of human γ globulin (Edelman *et al* 1960 Franklin and Stanworth 1961) By immunoelectrophoresis 2 antigenically distinct fractions can be identified One migrates more slowly than 7S γ globulin and therefore is called the S fragment The other migrates faster and accordingly is designated the F fragment The S fragment is analogous to pieces I and II of rabbit γ globulin it contains univalent antibody activity (Morse and Heremans 1962) The F fragment corresponds to piece III There is no immunologic cross reaction between the S- and the F fragments

This new approach has led to a better understanding of the structural basis for the well known immunologic interrelationship between the various γ globulins The cross reacting determinants are confined entirely to the S fragment and the F fragment is unique for 7S γ globulin (Franklin and Stanworth 1961) Thus the chemical groups that occur in all major classes of γ globulin have been identified as a part of the fragment which carries the antibody-combining site

Fragmentation into Polypeptide Chains Following the discovery of the critical role played by disulfide bonds in the structure of 19S γ globulin (Deutsch and Morton 1957) Edelman (1959) demonstrated their significance for the structure of 7S γ globulin Treatment of the protein in strong urea solutions with reducing agents resulted in diminution of the molecular weight from 160 000 to 50 000 The concomitant appearance of 14 to 18 sulfhydryl groups per molecule indicated that 7 to 9 disulfide bonds were broken in the process Edelman interpreted these findings to indicate that the 7S molecule is composed of several polypeptide chains and that these chains are interlocked by S S bonds and weaker forces

Further evidence for the validity of this hypothesis came from experiments employing starch gel electrophoresis (Edelman and Poulik 1961) Two different categories of fragments were detected in reduced and alkylated 7S γ globulins One category comprised electrophoretically fast migrating units which later turned out to be of very low molecular weight they were called light (L) chains The other consisted of more slowly migrating and heavier fragments they were termed heavy (H) chains The pattern of reduced whole γ globulin was not very revealing However the patterns of reduced myeloma proteins and antibodies disclosed the presence of only a small number of chains in a given protein Most interestingly starch gel patterns obtained on reduced individual γ globulins differed from one protein to the other leading to the impression that the individual species of 7S molecules contained similar or identical H-chains but varied in their set of L-chains Exactly how many chains make up one γ globulin molecule was difficult to ascertain Quantitation was seriously hampered because of insolubility of the dissociated chains outside strong urea solutions

Further progress was afforded by the development of a method which allowed preparation of the chains in a soluble form without the use of urea (Fleischman *et al* 1963) It was now possible to separate H and L-chains by a simple gel filtration process to quantitate their relative proportions and to determine the molecular weights Utilizing the body of accumulated information Porter (1962) proposed a model for the structure of rabbit 7S γ globulin (Fig 1) He envisions one molecule to consist of 4 polypeptide chains Two of these are H chains having a molecular weight of 50 000 to 60 000 and 2 are L-chains with a molecu

lar weight of 20 000 to 25 000 The heavy chains are held together by 3 S-S bridges and the light chains are linked each to 1 of the 2 heavy chains through 1 disulfide bond Thus according to Porter there exists a total of 5 interchain disulfide bonds per molecule However Palmer and Nisonoff (1964) have found only 1 S-S bridge between the 2 heavy chains and the total number of interchain disulfide bonds may be only 3

Papain is visualized to act on the heavy chains only In terms of the model piece III and F fragment correspond to the larger portion of the 2 H-chains pieces I II and S fragment correspond to the rest of the molecule consisting of the smaller portions of the split H chains plus L-chains

Of the 2 types of polypeptide chains L chains appear to have the greater structural significance Both molecular individuality as well as features shared by all γ globulins are expressed through them As indicated by starch gel electrophoresis of reduced antibodies and myeloma proteins individual species of 7S molecules differ primarily in their set of L-chains On the other hand these L-chains also carry the antigenic determinants that are characteristic for the entire family of γ globulins (Fahey 1963) Thus these polypeptide chains vary greatly among γ globulin molecules but must also contain regions that are constant in composition and identical for all γ globulin molecules

However the matter is even more complex There are 2 groups of light chains which are antigenically unrelated (Mannik and Kunkel 1963 Fahey 1963) In 7S γ globulin approximately 60 per cent of the molecules are endowed with L-chains of group I, and 30 per cent with those of group II An antiserum prepared against group I L chains will not react at all with group II molecules, and vice versa These types of L chain molecules are also found in 19S γ globulins β_A globulins and their pathologic counterparts (Mannik and Kunkel, 1962), as well as in γ_L globulin (Stevenson 1962) and Bence-Jones proteins In fact the 2 groups were originally discovered by Korngold and Lipari (1956) in Bence Jones proteins which consist entirely of light chains

In summary work on the chemical and the antigenic structure of γ globulin has led to the development of the following tentative concept The molecules of 7S and 19S γ

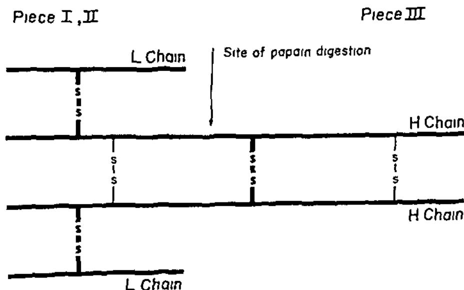


Fig 1 Structure of rabbit 7Sγ globulin as originally proposed by Porter (1962) who visualized three S S bonds linking the two H-chains and as modified by Palmer and Nisonoff (1964) who demonstrated that there is only one S S bond between the H-chains (In Porter s terminology H-chain = A-chain L-chain = B-chain)

globulin are composed of light and of heavy polypeptide chains The chains are interlocked by disulfide bonds γ_L Globulin (and Bence Jones proteins) consist of light chains only The heavy chains are specific for each class of γ globulin ie for 7S 19S γ globulin and for β_{2A} Within a given class there is little variation in heavy chain properties By contrast light chains exhibit no class specificity L-chains with the same general features occur in all 4 classes of γ globulin However they do possess group specificity giving rise to 2 categories of L-chains Each molecule of the γ globulin family belongs to either one or the other group depending on its L-chain make up In addition to these general structural features L-chains possess properties which confer individuality on a single species of γ globulin molecules thus giving rise to great molecular heterogeneity within the various classes

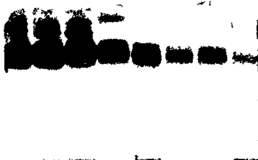


FIG 2 Starch gel patterns of reduced and alkylated guinea pig antibodies of different specificities (Edelman *et al* 1961) Direction of migration is toward the top The more slowly migrating bands represent H chains the faster ones L chains The first pattern on the left was obtained from whole guinea pig γ globulin The experiment demonstrates the variation in L chains between antibodies of different specificities

THE PROBLEM OF THE ANTIBODY COMBINING SITE

Theoretically specific antibody activity may be a function either of L-chains or of H-chains or of an interaction between both Since the S fragment of papain digested antibody behaves as univalent antibody it is evident that the larger portion of H-chains is dispensable for antibody activity and not involved in the active site What about the L-chains which are contained in S fragments? When purified antibodies against haptens are dissociated and examined by starch gel electrophoresis in urea discrete electrophoretic patterns are obtained (Fig 2) Antibodies of different specificities give rise to patterns which differ in the number and the distribution of the bands that represent L-chains However highly cross reactive antibodies yield patterns that resemble each other closely (Edelman *et al* 1961) It appears that antibodies of different specificities consist of different types of L-chains and that L-chains might therefore carry antibody combining activity However isolated L-chains proved to be completely inactive

Unlike L-chains purified H-chains from certain antibodies were found to be active in terms of specific inhibition of precipitation and in terms of co precipitation Recovery of specific activity was incomplete and varied with the degree of reduction (Fleischman *et al* 1963) Porter concludes that the combining region is located on H-chains and that the variability of L-chains is unrelated to antibody activity

However other recent reports indicate that L-chains although inactive *per se* bear an intimate relationship to the combining region Labeling of the combining site by different methods followed by dissociation of the antibody disclosed the presence of label in H and in L-chains (Metzger *et al* 1964 Roholt *et al* 1963) These observations suggest participation of L-chains in the formation of the active site although the extent of this participation and its significance for antibody activity remains unknown

Also unknown is whether the specificity of the combining region is determined by amino acid composition and sequence or by folding According to a hypothesis put forth

by Karush (1960), specificity is acquired by variable pairing of sulfhydryl groups and subsequent stabilization of conformation by disulfide bonds In this way a small number of disulfide bonds could afford the formation of a large number of different folding pat terns According to Buckley Whitney and Tanford (1963) this possibility appears to be eliminated by the available chemical data (Fleischman *et al* 1963) The number of distinguishable patterns which can be formed within the restrictions of the known ar rangement of intrachain S S bonds in pieces I and II is exceedingly small These authors conclude that antibody specificity is gen erated primarily by differences in amino acid sequence in some portion of the antibody molecule

COMPLEMENT

The term complement refers to a highly complex system of blood serum factors which possesses the potential capacity to cause ir reversible damage to cell membranes It con sists of at least 9 or 10 components the exact number being unknown It is activated by interaction with antigen antibody com plexes or γ globulin aggregates Activation is followed by a chain reaction which in volves all the components in characteristic sequence and results in loss of over all com plement activity By virtue of its combining power with γ globulin complement is capa ble of precipitating soluble antigen antibody complexes and of reacting with cell mem branes *For its cytotoxic action specific* antibody to cell membrane antigens is re quired in most instances The chemical mode of the complement reaction and the mecha nism by which complement interferes with normal membrane function are unknown

The Complement Reaction

The Components In the course of the discussion of antibodies it was possible to proceed from a mere description of serologic phenomena to an analysis of the underly ing molecular properties of antibody protein i e γ globulin A similarly illuminating ad vancement of our understanding of comple ment has not yet been achieved For most of the individual activities which together form the cytotoxic capacity of serum the chemi cal correlative is not known In the few in stances where it has been identified recently many of the physicochemical and chemical details have not been worked out Further with one exception none of these activities is defined in familiar biochemical terms They remain for the time being enigmatic serologic activities defined only by their role in the cytotoxic activity of fresh blood serum A comparison of the few isolated and puri fied complement components with each other and with γ globulin shows that complement components bear no antigenic resemblance to γ globulin and unlike different antibodies no resemblance to each other They appear to possess as different molecular properties as they have different functions in the com plement reaction

Originally complement activity was esti *mated by bacteriolysis* When immune hemolysis was discovered antibody-coated erythrocytes became the universally employed indicator system In most laboratories sheep erythrocytes are used and are sensitized with nonagglutinating quantities of antibody to sheep erythrocytes produced in rabbits The widespread use of erythrocytes as indicator cells has created the impression that comple *ment is primarily* hemolytic The fact is that a large variety of cells of mammalian origin as well as from lower animals and certain microorganisms are susceptible to the action of complement The advantage of using erythrocytes is that part of their intracellular protein is intensely colored and can b *readily quantitated spectrophotometrically* after liberation from the cells Therefore the amount of extracellular hemoglobulin is used commonly as a convenient measure for the number of red cells lysed by complement

That complement is not a single substance can be demonstrated easily by the separation of serum into euglobulins and pseudoglob ulins which is accomplished by dialysis of serum against buffer of low ionic strength Neither the euglobulins nor the pseudoglob ulins *per se* are capable of lysing antibody coated red cells However recombination of the 2 fractions results in reconstitution of the hemolytic activity of the original serum Treatment of serum with small amounts of ammonia or hydrazine as well as heating at

56 C for a short period of time also results in hemolytically inactive complement That the hydrazine sensitive material is not identical with the heat labile substance but that both are independent parts of the com plement system is shown by recovery of activity after pooling of the 2 differently treated sera In this and in similar fashion early students of complement succeeded in demonstrating that complement consists of at least 4 components with widely diverging properties These components were num bered and referred to as C'1 C 2 C'3 and C'4 C 1 and C 2 were found to be thermo labile and C 3 and C 4 thermostable com ponents C 1 and most of C'3 were charac terized as euglobulins C 2 and C 4 as pseudoglobulins C'3 could be preferentially inactivated by incubation of serum with zymosan which is an insoluble yeast poly saccharide and C 4 was destroyed by treat ment with ammonia or with hydrazine Analysis of the sequence of action of these 4 components in immune hemolysis estab lished that C 1 initiates the reaction and that this component is followed by the 4th the 2nd and finally the 3rd component

Only recently have the new and powerful protein separation methods been employed for the separation of complement activities After adsorption of serum to a DEAE cellu lose column the various complement compo nents are eluted in a characteristic sequence on variation of ionic strength or pH Applica tion of more complex chromatographic procedures has afforded the preparation of serum fractions which contain only one component Such preparations are referred to as functionally pure complement com ponents and have been used to great ad vantage in kinetic studies of the complement reaction (Mayer 1961a) It is important to realize at the outset that for the time being complement components are defined by their activities only because in most instances other parameters are unknown Therefore when a preparation of a component is con sidered to be functionally pure this does not imply and must not be confused with purity as defined by the criteria of protein chem istry In fact functionally pure components can be highly heterogeneous mixtures of proteins of which the active component con stitutes only a very small part

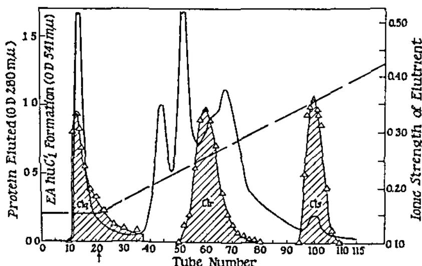


Fig 3 Separation of the classic first component of human complement into three subcomponents by DEAE chromatography (Lepow *et al* 1963) All three factors are required for generation of the activity of the classic first component It is an example of the molecular complexity of components previously believed to be monomolecular

In chromatograms of human or of guinea pig serum C 2 and C 4 activities can be de tected readily and are found in fractions which are definitely separable from each other (Rapp *et al* 1959 Becker 1960) However attempts to detect C'1 and C'3 activities have encountered considerable initial difficulty and have led subsequently to the realization that both C'1 and C'3 are complex each having a number of different activities C 1 of hu man serum is separated on chromatography into 3 subcomponents C'1q C'1r and C'1s (Lepow *et al* 1963) Recovery of whole C 1 activity is achieved only by recombina tion of the chromatographic fractions con taining the 3 subcomponents (Fig 3) Simi larly C 3 of guinea pig serum consists of 4 separate factors called C'3a, C'3b C 3c and C 3d (Linscott and Nishioka 1963) The new nomenclature of complement proposes to call these subcomponents of the classic 3rd component C'3 C'5 C'6 and C 7 accord ing to their sequence of action Provision has to be made for the discovery of additional factors essential for complement activity Indeed evidence has been obtained suggest ing the existence of at least one more com ponent which contributes to the activity of the classic 3rd component Details will be discussed below

The Reaction Steps Complement reactive sites on the cell surface are generated by the attachment of antibody molecules to mem brane antigens Complement binding ap parently requires that at least 2 antibody molecules must be brought together and held in close proximity to each other It is postulated that a site reactive with comple ment is formed either by 2 molecules of antibody of the 19S variety or by 4 of the 7S class (Weinrach and Talmage 1958) It follows that the degree of complement fixation should be dependent also on the distribution of antigenic sites on the cell surface If these are spaced so that antibody molecules will not attain the necessary degree of proximity interaction with complement would not be expected to occur The inability of Rh antibodies to fix complement has been explained in this fashion As further support for the above hypothesis the observation can be cited that the number of reactive sites on the surface of a sensitized cell is con siderably smaller than the total number of antibody molecules that have combined with it (Mayer, 1961a) To some extent anti body molecules are able to transfer from site to site and from cell to cell thus increasing the probability of formation of reactive sites and enhancing their hemolytic efficiency

The combining region for complement was originally attributed to fragment III of the antibody molecule (Taranta and Franklin 1961, Amiraian and Leikhim, 1961) After pepsin treatment which eliminates this part of the molecule complement fixation power is indeed strikingly reduced but is not com pletely abolished An appreciable proportion of the original fixation capacity is retained by the resulting 5S antibody derivative (Schur and Becker 1963) There is probably more than one region in the γ globulin molecule which is involved in interaction with comple ment The existence of at least 2 receptor sites has to be postulated one for the C'1 complex the other for C 4 since removal of C'1 from immune aggregates containing complement does not affect binding of C'4 Although the groups on γ globulin which are responsible for complement binding are unknown binding is dependent on an intact internal structure Reduction of disulfide bonds followed by alkylation a treatment which does not affect the molecular weight of 7S antibodies or their capacity to combine with antigen renders antibody molecules in capable of interacting with complement (Wiedermann *et al* 1963)

The first step of the complement reaction involves the components C'1q C 1r and C 1s and leads to the generation of esterase activ ity Upon uptake of the 3 components by re active sites the proesterase C 1s is activated presumably by the action of C'1q and C'1r (Lepow *et al* 1963) The product of this reaction EAC 1a is a cell (E) or a cellular site which contains antibody (A) and active first component (C 1a) and is ready to react with the next component Uptake of the whole C 1 complex requires participation of calcium ions although C'1q which will be discussed as 11S component has the ability to combine with γ globulin in the absence of bivalent cations This latter fact as well as moderate success in preparing the EAC 1q intermediate complex (Muller Eberhard

1961) has suggested a sequence of action which begins with C 1q and ends with C 1s Activated C'1 is able to transfer from site to site and from cell to cell as is typical of an enzyme it can react with many molecules of substrate (Borsos and Mayer 1962) When calcium is withdrawn from the reaction mixture the reaction between sensitized cells and the first component is reversed (Becker 1960) Under these conditions the fluid phase contains C'1 esterase as well as hemolytically active C 1 Although it is an enzyme, C 1 esterase is not involved in the actual process of cell lysis Its natural substrate is not the cell membrane but apparently 2 of the other complement components namely C 4 and C'2 Recent studies by Naff Pensky and Lepow (1964) indicate that the 3 subcomponents of C'1 occur in serum in the form of a macromolecular complex which is held together by Ca^{++} and has a sedimentation rate of 18S

The next event in the complement reaction is uptake by EAC 1a cells of C 4 activity a *step which depends entirely on the action of* C 1 esterase as evidenced by the failure of uptake on blocking of the esterase by DFP (diisopropyl fluorophosphate) Once C 4 activity is attached to the cell it is firmly retained even if subsequently C 1 is removed by treatment with EDTA (Becker 1960) Until recently it was not known whether uptake of C 4 activity corresponded to an actual attachment of the C'4 molecule or to an interaction with resulting change in membrane conditions and simultaneous destruction of C'4 activity in the fluid phase It has now been established that formation of EAC 1a 4 is dependent on physical uptake of the protein in which C'4 activity has been found to reside (Muller Eberhard and Biro 1963) This protein is called β_{1E} globulin and will be described below To combine with EAC 1a cells it must react with a receptor site located either on the antibody molecules or on the cell membrane since C 1 is excluded as a possible receptor While there is evidence for fixation of β_{1E} globulin to antibody in complement treated immune precipitates experiments to be alluded to later indicate that in cytolytic reactions β_{1E} globulin combines directly with the cell membrane

During the subsequent step C'2 reacts with EAC 1a 4 cells to produce intermediate complexes containing the activated form of the second component of complement C'2a This step requires magnesium ions In the somewhat awkward but systematic language adhered to by students of complement this product is symbolized by the expression EAC 1a 4 2a Two phases of this reaction can be distinguished during the first C 2 becomes loosely attached to EAC 1a 4 and during the second phase the activated first component acts on C 2 probably by cleaving the molecule and transferring an active group C 2a to a receptor on C 4 An inactive fragment C 2i is released into the fluid phase and can be demonstrated there by immunologic means (Mayer 1963) Activation which is highly dependent on temperature can be inhibited by agents which block or competitively inhibit C 1 esterase One of the peculiarities of C'2a is its marked lability which is particularly conspicuous at an elevated temperature and results in reversion of *the intermediate complex* EAC *1a 4 2a to the* EAC'1a 4 state (Mayer 1961a) The cause of the decay has not been elucidated It is prevented or perhaps it becomes undetectable and immaterial when the next 3 components of the complement system have completed their reaction with the cell Evidence is accumulating suggesting that C 2a constitutes an enzyme or at least part of an enzyme which acts on C 3

Conversion of an EAC 1a 4 2a cell to a state in which it will undergo spontaneous lysis is known to require at least 4 factors of guinea pig serum (Linscott and Nishioka 1963) Two of these are thought to be necessary to render an EAC'1a 4 2a cell unsusceptible to the effects of time and temperature i e to preserve its reactivity with the last 2 components of the system Although such an accumulation of factors may seem to be elaborate enough to achieve and to secure a simple biologic effect such as immune cytolysis the latest evidence indicates that actually 3 separate factors are required for the conversion of EAC 1a 4 2a to a stable intermediate thus at least 5 factors are necessary for the conversion of the same complex to an irreversibly damaged cell that finally will undergo lysis

The component which is able to react directly with EAC'1a,4 2a cells is defined as C'3 and has been identified with a protein in human serum designated β_{1C} globulin In the course of their interaction with cells containing C'2a a certain proportion of C'3 molecules is taken up and firmly retained by cellular sites while the majority of the molecules are released into the fluid phase in physicochemically altered and hemolytically inactive form Both uptake of C 3 and conversion to C 3i are highly temperature dependent processes they are independent of bivalent cations and are not catalyzed by C'1 esterase

Following C 3 two factors here referred to as C 5 and C'6, are the next ones to act Only if both of these components react with EAC'1a 4 2a 3 will an intermediate complex be formed which no longer displays the decay phenomenon so characteristic for its 3 immediate precursors (Nilsson and Muller Eberhard 1964 Nelson personal communication Wellensiek and Klein 1965) The stable complex may be represented by the expression EAC 1a 4 2a 3 5 6 It is characteristic for C'5 and C'6 that they require for their action the presence on the cell of both C 2a and C 3 If C 2a has decayed cells now in the state EAC'1a 4 3 can be restored to EAC'1a 4 2a 3 by addition of C 2 (Linscott and Nishioka 1963) C'5 of human serum has been identified with a protein which, on the basis of its immunoelectrophoretic behavior has been named β_{1F} globulin (Nilsson and Muller Eberhard 1964)

The final 2 steps lead to the critical changes in membrane integrity which initiate cell lysis After action of C 7 the cells exhibit increased fragility and this has been interpreted to indicate partial disintegration of cell membrane structure without breakdown of membrane function The fatal event is caused by C'8, after it has acted on the cells lysis ensues Heat lability of C 7 and C 8 and temperature dependence of the final reaction suggests that these 2 components may be enzymatic in nature (Linscott and Nishioka 1963) Since questions concerning nomenclature have not been settled definitively it should be pointed out that the terms C'3 5 6,7 8 used by the author correspond to C'3c b e a d of Nelson s terminology

The entire sequence of events is illustrated diagrammatically in Figure 4 Although there is reason to believe that an organism endowed with this highly complex cytotoxic mechanism has a selective advantage over one that lacks it one cannot help wonder why it is so unusually complex To our mind the biologic role of complement is revealed in its capacity to impair cell membranes Experimentally destruction of cell membranes in vitro and in vivo is accomplished readily by enzymes such as lecithinase and, indeed enzymatic activity is implicated in the final steps of complement action Why then has nature evolved and maintained this elaborate system when the task could be achieved essentially by a single enzyme? Evidently to be compatible with life cytotoxic activity must not occur in a

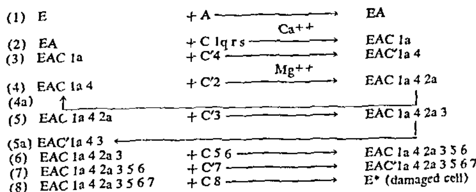


Fig 4 Complement reaction sequence

form which would act indiscriminantly Safety devices are required which impose rigid restrictions on its action and direct it to selected targets The components which precede the last 2 in the complement reaction may well represent such a device On the other hand one has to be mindful of the possibility that earlier steps of the complement reaction may have their own biologic significance which may be entirely unrelated to membrane damage As will be discussed below complement promotes phagocytosis for this to occur the reaction does not have to go to completion Similarly other as yet unidentified biologic functions may be associated with limited parts of the complement reaction or with individual components

Chemical Aspects

C'1 Esterase The discovery of the enzymatic nature of C 1 constitutes perhaps the most significant recent contribution in the complement field With this observation complement has come within reach of biochemical definition

The initial observation was made by Levine (1955) who found that diisopropyl fluorophosphate (DFP) a powerful inactivator of esterases inhibits immune hemolysis by guinea pig complement He also found that this effect is due to an action of DFP on EAC'1a 4 cells Subsequently Becker showed the affected component to be C 1a From his work (Becker 1956 1960) and that of Lepow (Lepow *et al* 1956 Ratnoff and Lepow 1957 Lepow *et al* 1963) the following concept evolved A portion of the first component C'1s represents an inactive proenzyme which cannot be inhibited by DFP Upon *interaction with C 1q and C 1r* as it occurs during immune hemolysis the proenzyme is activated and esterase activity ensues Unlike its precursor the activated enzyme is readily blocked by DFP as evidenced by disappearance of esterase activity and of C'1a hemolytic activity

The enzymatic activity of C'1a can be demonstrated with the aid of certain synthetic amino acid esters which are hydrolyzed by C'1a at the ester linkage For human C 1 esterase the most susceptible compound is N acetyl L tryosine ethyl ester p toluene sulfonyl L arginine methyl ester and benzoyl L arginine methyl ester also display a high degree of susceptibility whereas many other compounds of similar structure do not

Although no proteolytic activity has been demonstrated for C'1 esterase the enzyme is capable of destroying C 2 and C'4 activity in serum This may seem to be in striking contrast with its presumed function the catalysis of the union of C'4 and C 2 with antibody coated cells However if it is assumed that C 1 esterase acts identically in both cases for instance by splitting a bond which thereafter can react either with a cellular receptor site or with water then the discrepancy is resolved Whether a molecule is activated or inactivated by C 1 esterase may thus depend merely on the immediate availability or the lack of cellular receptor sites Much is in favor of such a hypothesis

For activation and uptake of C'1 calcium ions are important The role of calcium during activation is strictly specific (Mayer 1961) whereas a number of bivalent cations can substitute for calcium in establishing and maintaining the bond between C 1a and EA (Wirtz and Becker 1961) It is possible that calcium functions as a ligand linking 2 negatively charged molecules

More than 2 400 fold purification of C'1 esterase has been achieved by chromatographic technics (Haines and Lepow 1963) However these highly purified preparations are still not quite homogeneous in the ultracentrifuge and on immunochemical analysis The enzyme migrates as α globulin on paper electrophoresis and has an s rate of 4S

11S Component. When human serum is adsorbed with soluble aggregates of γ globulin in the presence of EDTA hemolytic complement activity is lost When this observation was first made it was most startling and at variance with the well established concept that complement cannot interact with immune aggregates in the absence of bivalent cations In confirmation of this widely held view it was found that none of the classic components was removed by this treatment. Therefore it was concluded that a factor had been eliminated from serum which although not a classic component is nevertheless essential for hemolytic complement activity and is capable of combining directly

with γ globulin even in the absence of cal cium ions This postulated factor was isolated from γ globulin aggregates which had been exposed to EDTA containing serum (Muller Eberhard and Kunkel, 1961)

Since the sedimentation coefficient of this new complement component is 11 1S it was tentatively called the 11S component Its electrophoretic behavior resembles that of a slow γ globulin it must be one of the most basic proteins of human serum Although it migrates in the electric field like a γ glob-ulin it bears no immunochemical relationship to γ globulin

When purified 11S component is added to a serum preparation previously depleted of this protein and thus rendered hemo-lytically inactive activity is fully restored The quantity of 11S component required for full restoration of activity corresponds to approximately 0 05 per cent of the total serum protein present in the reaction mix ture Interaction of the component with soluble immune or γ globulin aggregates results in their precipitation suggesting at least 2 combining sites on a molecule of 11S component Both hemolytic and precipitating activity are rapidly destroyed on heating of the protein to 56° C The bond between 11S component and antibody coated cells or soluble γ globulin aggregates appears to be weak It is much strengthened by the par ticipation of C 1r and C′1s

Lepow recently has shown that the 11S component which he calls C′1q together with C 1r is required for activation of C′1 esterase Thus present evidence suggests that the 11S component assumes the important function of initiating the complement reaction by combining with γ globulin molecules and by participating in the activation of C 1 ester ase An analogous activity has been described for rabbit and for guinea pig serum (Bar baro 1963)

β_{1E} Globulin This is the 4th component of human complement and the 1st of the classic components which has been isolated and characterized Isolation was accomplished by a combination of preparative electro phoresis and chromatography (Muller Eber hard and Biro 1963) The final product exhibited a high degree of physicochemical homogeneity and could be defined with the aid of antisera as a single immunochemical entity of human serum The protein was designated β_{1E} globulin It has a sedimenta tion coefficient of 10S which is independent of concentration It contains a carbohydrate moiety On treatment with dilute hydrazine which is known to inactivate C′4 the protein undergoes some distinct physicochemical changes

Before concluding that an activity resides in a given protein, it is necessary to demon strate their inseparability by a variety of separation procedures In addition whenever possible more specific criteria should be employed In the case of β_{1E} globulin such criteria are given by the known behavior of C′4 activity In complete analogy to C′4 activity β_{1E} globulin combines only with cells containing the active first component of com plement and not with sensitized cells or with DFP inhibited EAC 1a Further treatment with hydrazine renders β_{1E} globulin incap able of combining with EAC 1a cells but when EAC′1a 4 cells, which contain β_{1E} are treated with hydrazine β_{1E} remains cell bound as does C 4 activity The presence of β_{1E} globulin on the cell surface can be demonstrated either directly by using radio actively labeled β_{1E} or indirectly by aggluti nation of the cells with an antiserum to this component It thus appears that β_{1E} globulin represents human C′4 and that the conver sion of EAC 1a to EAC′1a 4 cells corre sponds to attachment of β_{1E} to the cells

β_{1C} Globulin Only after its isolation from human serum was this protein recognized as an essential part of the hemolytic comple ment system (Muller-Eberhard *et al* 1960) It is defined as C′3 because it interacts directly with cells containing C′2a (EAC 1a 4 2a) The reaction does not proceed at 0 C The protein migrates in the electric field as a slow β_1 globulin it has a sedimen tation coefficient of 9 5S is endowed with a carbohydrate moiety and is characterized by a pronounced lability On treatment with dilute hydrazine or ammonia or on aging it undergoes distinct physicochemical changes The altered protein has a greater electro phoretic mobility and a lower sedimentation rate (7S) it is designated β_{1A} globulin In this form the protein is hemolytically inactive

TABLE 2 PROPERTIES OF ISOLATED COMPLEMENT COMPONENTS

	11S COMPONENT	β_{1E} GLOBULIN	β_{1C}-GLOBULIN	β_{1F} GLOBULIN
20 w	11 1S	10 0S	9 5S	9 4S
Electrophoretic mobility	γ	β_1-α_2	β_2 β_1	β_1-α_2
Serum concentration (µg /ml)	20 30	30 50	300-400	30 50
Activity	C'1q	C'4	C'3	C 5
Receptor	EA	EAC'1a	EAC'1a 4 2a	EAC'1a 4 2a 3
Temperature requirement	0	0	+	
Inactivated by	heat	N_2H	N H	heat

The conversion from the active β_{1C} to the inactive β_{1A} form can be followed best by immunoelectrophoretic analysis of whole serum or of the isolated protein It is so conspicuous that this phenomenon originally led to the recognition of the protein as a potential complement component

During immune hemolysis when C 3 activity is consumed β_{1C} globulin is affected in seemingly two ways it is changed and it is in part attached to the cells Approximately 80 to 90 per cent of the protein is found after the reaction in the fluid phase It is present in an electrophoretically faster form closely resembling β_{1A} globulin but differing in that its sedimentation rate is practically unchanged To distinguish this reaction product from β_{1A} globulin the product of aging the term β_{1G} globulin is tentatively proposed Approximately 10 to 20 per cent of the original β_{1C} molecules are found to be in firm combination with the cell membranes Whether these molecules are in the β_{1C} or in the β_{1G} state or represent still another form is not known with certainty Some observations suggest that the protein occurs on the cell surface in more than one discrete form and that the form which is reactive with C 5 is β_{1G}

The chemical events underlying the conversion of β_{1C} globulin during the complement reaction have not yet been elucidated However since the phenomenon is temperature dependent and highly reproducible it may be postulated that it is mediated by an enzyme associated with the complement system When cells in the state EAC 1a 4 2a are reacted at 37 C with highly purified human β_{1C} globulin (free of detectable quantities of C'5 and C'6) conversion proceeds rapidly Since DFP inhibits neither uptake nor conversion and since cells previously decayed to the EAC'1a 4 state are completely inert to β_{1C} globulin it must be concluded that C'2a is responsible for uptake and conversion of β_{1C} globulin (Muller Eberhard *et al* 1964) The possible enzymatic nature of C'2a deserves serious consideration and exploration

β_{1F} Globulin This protein has been recognized only recently as a new entity of human serum and as the carrier of C'5 activity (Nilsson and Muller Eberhard 1964) Detection was faciliated by an antibody occurring in some antisera to β_{1C} globulin The original preparations of β_{1C} globulin were contaminated with small amounts of β_{1F} globulin Electrophoretically the protein behaves like a fast β globulin its sedimentation coefficient is 9 4S Its activity is exceedingly thermolabile

Table 2 summarizes a few characteristic features of the components isolated so far In spite of certain physicochemical similarities these proteins are completely unrelated to each other by immunochemical criteria They also are unrelated to γ globulin

OTHER HUMORAL FACTORS

In addition to antibody and complement some other factors may play a role in humoral defense mechanisms

LYSOZYME

This is an enzyme which is capable of lysing certain bacteria by attacking cell wall mucopeptides It is widely distributed in nature and occurs in the body fluids including serum and plasma of man and animals



FIG 5 Immunoelectrophoretic demonstration of two complement components in human serum The single precipitin line above represents B_{1C} globulin (C 3) it was obtained by filling specific anti B_{1C} antiserum into the longitudinal well at the top The line at the bottom represents B_{1E} (C 4) it was developed using a specific anti B_{1E} antiserum The trough in the middle was filled with antiserum to whole human serum

The richest source for lysozyme is egg white

Lysozyme is a basic protein with an iso electric point at pH 10 11 Its sedimenta tion coefficient ^{s} 20 w is 1 9S the molec ular weight 14 400 (Sophianopoulos *et al* 1962) It is stable at acid pH even when heated but unstable in alkaline pH The primary structure of egg white lysozyme has been worked out by Jolles *et al* (1963) who also showed that there is 1 disulfide bridge in the molecule

Lysozyme activity is commonly deter- mined by lysis of a gram positive coccus which was isolated by Fleming and named by him *Micrococcus lysodeikticus* Degra- dation of this microorganism by lysozyme results in the release of N acetylamino sugar compounds and N acetylamino sugar pep- tide complexes Lysozyme breaks the gly cosidic bond between carbon atom Number 1 of N acetylmuramic acid and carbon atom Number 4 of N acetylglucosamine (Salton and Ghuysen 1959) accordingly the en zyme is a β (1 4) N acetyl-hexosaminidase

BETA LYSIN

Beta lysin (Petterson, *1926*) also called the beta bactericidal system of serum is distinct from antibody and complement and also from lysozyme It is capable of killing a number of gram positive microorganisms by an unknown mechanism of action *Bacil lus subtilis* is commonly used to test for its presence in serum Beta lysin can be separated into two components which are both required for full bactericidal activity against *Bacillus subtilis* (Myrvik and Leake 1960) Com ponent I is precipitated at 16 per cent ethanol concentration and component II at 40 to 50 per cent Both components are heat stable and nondialysable neither is inactiv ated by ammonia The activity is potentiated by bicarbonate Heat stability distinguishes the system from complement components C'1 and C'2 resistance to treatment with am monia from components C'3 and C'4 Since addition of specific agglutinins to serum does not enhance the activity antibody is believed not to be involved Lysozyme from egg white cannot substitute for either component While quite a bit is known about what beta lysin is not little in fact is known about what it is Some light on its peculiar nature is shed by the finding that plasma even when pre pared without resort to anticoagulants mani fests no such bactericidal activity apparently the responsible factors are liberated from platelets during the clotting process (Hirsch 1960 Jago and Jacox 1961)

OPSONIN

Substances present in blood serum which act on bacteria to render them subject to phagocytosis are called opsonins They func tion by altering the surface conditions of ob jects to be ingested There appears to be a

number of substances in serum that can act as opsonin Antibody exerts a strong opsonizing effect in many instances and complement generally enhances this effect Phagocytosis of larger cells e g erythrocytes proceeds poorly or not at all in the absence of complement In certain instances still other serum factors come into play Hirsch has described a heat labile opsonin which is operative when specific antibody is lacking It is distinguished from antibody by the following properties it is not a γ globulin and it lacks specificity it is heat labile and is inactivated by dilute ammonia and hydrazine it reacts rapidly with bacteria at 38° C but not at 0 C (Hirsch and Strauss 1964) These properties are reminiscent of those of complement However the heat labile opsonin described by Hirsch is distinct from hemolytic complement in that it does not require calcium and magnesium for combination with bacteria The presence of this material on the surface of opsonized and washed bacteria can be demonstrated by the fluorescent antibody technic (Hirsch 1964) The chemical or immunochemical nature of the heat labile opsonin is unknown

Humoral Factors in Cellular Immune Mechanisms

Mention should be made of a group of humoral principles which are involved in cellular immune and defense mechanisms Hardly anything is known about their nature and practically nothing about their mode of action However the biologic role of these factors is so important that attention should be focused on them

A number of years ago Lawrence discovered that delayed sensitivity to bacterial and fungal antigens can be transferred from immune to nonimmune individuals by leukocyte extracts The active principle which originates from lymphoid cells was called transfer factor It is dialysable and has a molecular weight of less than 10 000 Its activity is resistant to treatment with DNAse and RNAse The factor is released on contact of sensitized lymphoid cells with the antigen The significance of transfer factor derives from its ability to endow recipients with delayed sensitivity *de novo* In other words this factor is capable of transferring immunologic information and of causing the recipient to possess the immunologic memory of the donor for prolonged periods (Lawrence 1960)

Recently Najarian and Feldman (1963) demonstrated the transfer of homograft immunity by a soluble substance which was obtained from disrupted tissue sensitized lymphoid cells The material possessed immunologic specificity and was not identical with transfer factor Thermostability solubility and chromatographic characteristics suggested to these authors that it might be γ globulin representing transplantation antibody

Another recent and most interesting development is the demonstration by Levey Trainin and Law (1963) of a humoral factor that diffuses from thymic tissue and appears to be responsible for the evolution of immunologically competent lymphoid tissue Mice thymectomized within 12 hours after birth develop the characteristic symptoms of neonatal thymectomy marked lymphopenia involution of lymphoid tissue and severe wasting disease Death occurs at 7 to 8 weeks of age In contrast these symptoms did not develop when a cell tight diffusion chamber containing isologous newborn thymus was implanted in 3 to 4 week-old thymectomized mice These animals did not show depletion of lymphocytes or involution of lymphoid tissue or any signs of wasting disease Independently Osoba and Miller (1963) demonstrated that a humoral factor of embryonic or neonatal thymus is able to restore the capacity to reject homografts in thymectomized mice

Elucidation of the nature of this group of humoral factors will contribute considerably to the advancement of our understanding of immune phenomena

EFFECT OF ANTIBODY AND COMPLEMENT ON CELL MEMBRANES

Effect of Antibody

Effective immune cytolysis requires both antibody and complement To evaluate critically the role of complement it is necessary to determine whether antibody alone might be capable of inducing some changes in cell

metabolism or morphology Easton, Goldberg and Green (1962) have studied this question extensively, utilizing Krebs s ascites tumor cells Viewing the cells with the electron microscope they found that ferritin labeled cytotoxic antibody was localized on the outer cell membrane None of the ferritin label was detected inside the cell, indicating that antibody cannot pass the membrane of intact cells Even over prolonged periods of time antibody had no effect on the cytoplasmic structures mitochondria and endoplasmic reticulum However it did induce folding of limited portions of the outer cell membrane particularly in those areas where the antibody concentration was especially dense These folded areas appeared to facilitate agglutination, since interdigitation of surface projections of adjacent cells was observed frequently The morphologic change induced by antibody was not accompanied by any functional lesion In fact the cells could be grown in normal fashion with cytotoxic antibody present These results support the concept that cytotoxic antibody alone has no adverse effect on cells and that its function in immune cytolysis is to mediate the action of complement Further evidence of the mediating role of specific antibody is provided by observations indicating that antibody can be replaced by so-called nonspecific sensitizers

Nonspecific Sensitizers

A number of substances which chemically have nothing in common with antibody have been shown to function as sensitizers in immune hemolysis The most thoroughly studied is polyethylene glycol Cowan (1954) demonstrated its ability to replace antibody in the conventional hemolytic system consisting of sheep red cells rabbit antibody and guinea pig complement For effective hemolysis the concentration of polyethylene glycol in the reaction mixture had to be approximately 6 per cent and the concentration of calcium 5 times the conventional value Under these conditions the reaction resembled in all phases the ordinary type of immune hemolysis which depends on antibody Since cells treated with polyethylene glycol do not remain sensitized during subsequent washing Cowan concluded that this substance functions as a sensitizer by forming a loose, reversible link between the cell surface on the one hand and complement on the other

An alternative mechanism of action is suggested by the finding that polyethylene glycol causes reversible aggregation of γ globulin in whole serum (Dalmasso and Muller Eberhard 1964) These aggregates are capable of interacting with complement Therefore, it appears probable that polyethylene glycol causes γ globulin in the reaction mixture to aggregate and then binds some of the aggregates loosely to the cells Complement reacting with aggregates which are in close proximity to the cell surface thus may affect the cell membrane directly Whether processes similar to these in vitro experiments can occur in vivo is unknown

The use of polyethylene glycol has aided the exploration of complement-cell membrane interaction substantially Cells first treated with polyethylene glycol and human serum lacking cytotoxic antibody and then thoroughly washed were found to be coated heavily with β_{1E}- and β_{1C} globulin As judged by negative agglutination reactions with specific antisera these cells did not contain any of the 3 γ-globulins 7S γ globulin 19S γ globulin, β_A globulin Therefore it appears that the continued presence of these 2 complement components on the cell surface is not dependent on a sensitizer antibody or γ globulin It must be postulated that both components are capable of establishing a direct link with membrane sites

Nature of Lesion Caused by Complement

Morphology of Lysis of Mammalian Cells Goldberg and Green (1959) have conducted electron microscopic studies on Krebs s ascites tumor cells treated with specific antibody plus complement Within 2 minutes at 37° C dramatic morphologic changes become observable The cells begin to swell and large areas of the cytoplasmic matrix are cleared of all particulate structures The small electron-dense particles which form the background density of normal cells disappear Mitochondria and the endoplasmic reticulum also swell and give rise to the formation of large cytoplasmic vesicles All

these changes occur in the absence of detectable defects in the integrity of the outer cell membrane However Borsos Dourmashkin and Humphrey (1964) using negative staining with potassium phosphotungstate were able to demonstrate holes in the membranes of a variety of cells which had been lysed by antibody and complement The holes had a diameter of 80 to 100 A

The Permeability Defect The morphologic changes are accompanied by a drastic redistribution of water small ions and macromolecules between the cytoplasm and the outer fluid Within 5 minutes after the onset of complement action 90 per cent of the intracellular potassium has left the cells and extracellular sodium has entered them At this time only 15 per cent of intracellular protein has escaped Later after 1 or 2 hours 50 per cent of intracellular protein 75 per cent of RNA and most of the amino acids and the nucleotides have leaked into the supporting medium (Green *et al* 1959)

A thorough study of the escape rate for small and for large molecules has enabled Green Barrow and Goldberg (1959) to describe the course of events of cytolysis in the following way the action of antibody and complement gives rise to functional holes of small size in the membrane which permit free exchange of potassium and sodium between the interior and the exterior of the cell Equilibration results in an increased intracellular osmotic pressure The reason for a rise in intracellular pressure is the high concentration of cytoplasmic macromolecules With rising osmotic pressure following the action of antibody and complement water enters the cells and they swell Concomitant stretching of the cell membrane causes the primary lesions now to permit passage of macromolecules Swelling of the cells and subsequent loss of macromolecules can be prevented by a high concentration of extracellular protein sufficient to balance the intracellular colloid osmotic pressure Sucrose does not prevent swelling it can pass through the initial lesions into the interior of the cells Thus immune cytolysis represents a form of colloid osmotic lysis The nature of the initial lesion is still obscure

Formation of Spheroplasts from Gram Negative Microorganisms In the case of bacterial cells the primary injury caused by antibody and complement appears to involve the cell wall not the membrane The plasma membrane of bacteria is not immediately accessible since it is located on the inside of the cell wall The firm structure of the wall serves to prevent the cells from bursting in hypotonic environments For bacteria most environments are hypotonic because of their own high intracellular osmotic pressure Theoretically complement might destroy bacteria either by damaging the cell wall thereby depriving the underlying membrane of the much needed mechanical support, or it might impair the functional integrity of the plasma membrane causing leakage of intracellular constituents through an unharmed cell wall The latter alternative would require that the intact cell wall be permeable to macromolecules of the size of complement components

Recently several groups of investigators have studied the primary events in bacteriolysis induced by antibody and complement They found that lysis of bacteria can be prevented by high concentrations of sucrose or lactose in the medium Under these conditions the organisms convert to protoplast like osmotically fragile forms which remain viable as long as osmotic protection is provided The phenomenon has been demonstrated for *Shigella dysenteria* (Michael and Braun 1959) *Salmonella typhosa* and *Escherichia coli* (Muschel *et al* 1959) and for *Vibrio cholerae* (Freeman *et al* 1963) Evidently the action of complement is directed against the cell wall not the plasma membrane There is some evidence that the cell wall is not completely removed by complement but is sufficiently altered as to allow the cell to assume a spherical shape

Penicillin in the presence of sucrose also causes the formation of spheroplasts from gram negative organisms However the mode of action of complement and of penicillin differs (Michael and Braun 1959) Penicillin is most effective on bacteria which are in the exponential phase of growth complement is effective on bacteria in the stationary phase Penicillin interferes with cell wall synthesis complement appears to act enzymatically on cell walls previously formed

The One Hit Theory The number of

lesions required for lysis of a cell by complement is undoubtedly a reflection on the nature of the injury inflicted For immune hemolysis, Mayer (1961b) has postulated a one-hit or single site reaction mechanism His theory proposes that the production of a single damaged site is sufficient for lysis This is difficult to comprehend in view of the limited size of the membrane defect caused by complement It would seem to be reasonable and biologically sound to assume that a cell can compensate for a single lesion which is so small as to allow only electrolytes to pass freely The one hit theory was developed by Mayer when he found that the absolute velocity and the extent of the hemolytic reaction are independent of the total number of cells in the reaction mixture The observation precluded the possibility of a multiple hit or cumulative damage mechanism However it does not necessarily exclude the possibility that more than one molecule of any one of the complement components may interact with a single cellular site What the theory requires is that a single molecule of at least one of the components involved in the hemolytic reaction suffices for the production of a damaged site i e for at least one of the reaction steps there should exist a direct proportionality between the amount *of the complement component reacting* and the number of sites produced According to Borsos Rapp and Mayer (1961) this condition is fulfilled for the reaction between EAC'1a 4 and C'2 and according to Hoffmann (1960) for the interaction of EA with C 1 and of EAC 1a with C 4 A satisfactory correlation was found to exist between the number of lesions predicted on the basis of the one hit theory and the lesions actually counted by electronmicroscopy (Borsos Dourmashkin and Humphrey 1964)

Mode of Action of Complement

Three Hypotheses The injury caused to cell membranes by complement may be due to mechanical stress or direct enzymatic attack or to a nonenzymatic cytolytic agent produced in the course of the complement reaction The first two hypotheses imply a direct and the last an indirect mode of action of complement on cell membranes

The hypothesis of indirect action through a cytolytic agent is old but has been revived recently and tested experimentally by Fischer and Haupt (1961) These authors found that when fresh serum is treated with antigen antibody precipitates, a cytolytic substance is produced The material was isolated and identified by paper chromatography as lysolecithin While a protein free filtrate of fresh guinea pig serum contained only 0 5 μg of lysolecithin per ml, 3 7 μg/ml were found after incubation of serum with immune complexes A similar increment in the lysolecithin content of serum was not observed when any one of the classic complement components was inactivated prior to treatment The authors conclude that lysolecithin represents the end product of the complement reaction and the substance to which complement owes its cytotoxic capacity

The lysolecithin hypothesis implies that one of the two terminal complement components is a lipoprotein capable of donating lecithin and the other an enzyme which is able to convert lecithin to lysolecithin However a serious difficulty arises from quantitative considerations According to Fischer and Haupt, $3\ 85 \times 10^{10}$ molecules of lysolecithin are necessary for lysis of a single human erythrocyte Accordingly only 10^5 erythrocytes should be lysed by the amount of lysolecithin liberated from 1 ml of serum whereas the total hemolytic capacity of this amount of serum is far greater Therefore, it was postulated that the efficiency of lysolecithin is much increased when it is liberated at a critical site on the cell surface On the basis of present experimental evidence the lysolecithin hypothesis can be neither refuted nor accepted

Mechanical stress appears to be the least likely cause of the effect of complement on membranes No doubt a considerable number of complement molecules accumulate at a single reactive site therefore the possibility of increasing mechanical stress is by no means excluded but it seems unlikely to be the primary cause of membrane injury A cell in the state EAC'1a 4 2a 3 5 6 probably has taken up all the complement macromolecules which are capable of permanent attachment since C'7 and C'8 which are not depleted in immune reactions (Linscott and Nishioka 1963), probably are not

bound Yet such a cell does not display any tendency to lyse

Marked thermolability of C7 and C8 (Linscott and Nishioka 1963) and lack of their depletion in immune reactions are consistent with the assumption of an enzymatic mode of complement action

The "Substrate" Plasma membranes are thought to be composed of a bimolecular leaflet of lipid covered on both surfaces with a layer of protein Such a concept is consistent with the structure of membranes as observed in high resolution electron micrographs (Stoeckenius 1962) Accordingly there are at least 2 possible substrates for complement lipid and protein

Observations on immune bacteriolysis suggest that complement acts on lipid or lipopolysaccharide complexes Gram positive bacteria are generally resistant to destruction by complement their cell wall is deficient in lipid Gram negative microorganisms may be killed and lysed by antibody and complement A large proportion of their cell wall consists of lipid complexes Moreover lysis of gram negative organisms by complement can be inhibited by certain phospholipid compounds (Ginsburg 1960) Therefore the assumption may be justified that gram negative organisms and mammalian cells are susceptible to complement because of the lipid moiety in their wall and membrane respectively

Further evidence for the lipid nature of the substrate of complement has come from work on immune inhibition of the electron transfer system (Davis *et al* 1963) Antibodies against rat mitochondria have *per se* very little effect on the activity of dihydro diphosphopyridine nucleotide (DPNH) oxidase However marked inhibition is observed on addition of complement to the system Since DPNH-oxidase consists of a complex chain of oxidation reduction components it is not immediately evident with which component complement is interfering The electron transfer chain includes cytochrome C which functions as an electron bridge between the 3rd and the 4th component in the chain (Green and Fleischer 1963) Apparently it is here that complement exerts its effect because the complement inhibited system can be reactivated by the addition of small amounts of cytochrome C

The components of the electron transfer system including cytochrome C have been shown to occur in the form of complexes with phospholipids (phosphatidylcholine phosphatidylethanolamine phosphatidylinositol and polyglycerol phosphatide) These compounds are of vital importance for the activity of the electron transfer system since activity is lost when they are extracted and is regained when they are replaced (Green and Fleischer 1963) In view of the importance of phospholipids to the system it may not be too surprising that phospholipase A is able to inactivate DPNH-oxidase As in the case of inhibition by complement the effect of phospholipase is overcome by exogenous cytochrome C Phospholipase presumably cleaves the phospholipid-cytochrome C complex (Ambe and Crane 1959) Since phospholipase is also capable of rapidly lysing mammalian cells it may be concluded that the action of complement resembles in certain respects that of phospholipase

HUMORAL MECHANISMS OF HOST DEFENSE

Neutralization of Viruses and Bacterial Toxins

The capacity of viruses to infect cells and to multiply is readily inactivated by specific antibody Virus is accessible to antibody only as long as it is outside cells Antibody is not virucidal i e it does not destroy virus particles Instead antibody interferes with the attachment of virus to potential host cells There are 2 phases of virus attachment the initial reversible binding and the subsequent formation of an irreversible bond between virus and cell surface Whereas antibody does not interfere with reversible attachment of virus it does block the reaction leading to irreversible attachment Since the latter is a prerequisite for penetration and invasion of the host cell a neutralized virus particle is rendered incapable of injecting its constituents into the cell

Not all antivirus antibodies possess the capacity to neutralize Antibody to bacteriophages for instance may be directed against either the head or the tail antigen Since a phage particle combines with a host cell through groups located at the tip of its tail

only antitail antibody is able to neutralize the phage The surface of the phage tail is visualized to contain a repeating pattern of antigenic structures which enables it to accommodate a large number of antibody molecules However neutralization of a phage particle is effected by only a very few antibody molecules in fact a single molecule may suffice This is inferred from kinetic studies which indicate that the rate limiting step is the reaction of 1 antibody molecule with 1 virus particle (Cann and Clark 1956) Various aspects of the problem of virus neutralization by antibody have been discussed by de St Groth (1962) and by Talmage and Cann (1961)

In addition to immune antibody normal mammalian sera of various sources have been reported to possess virus neutralizing activity Recently several groups of investigators (Cowan 1962 Muschel and Toussaint 1962) studied the inactivation of coliphages by normal sera These authors found that normally occurring specific antibody as well as complement participates in the reaction Unlike immune antibody normal serum antibody is *per se* not effective against viruses although it does combine with phage particles even in the absence of complement Neutralization occurs only when complement and bivalent cations are present The observed complement requirement suggested a mechanism of neutralization for normal serum which differs from that ascribed to immune antibody However the possibility of a virucidal action of complement could be excluded Inactivation by normal serum is completely reversed on treatment of neutralized phage with papain (Muschel and Toussaint 1962)

A number of animal viruses have also been shown to be inactivated by normal serum In certain cases serum substances other than antibody and complement have been held responsible for the effect These substances have been discussed by Ginsberg (1960)

Little is known about the mechanism of neutralization of bacterial toxins although there are indications that it is similar to the neutralization of viruses Many pathogenic species of gram positive bacteria produce exceedingly poisonous protein exotoxins (see Chap 7) Antitoxin appears to exercise its neutralizing effect by preventing the interaction between toxin molecules and susceptible sites in the tissues How many antitoxin molecules are needed for neutralization of one molecule of toxin has not been established unequivocally As in the case of viruses neutralization is reversible by dissociation of the toxin antitoxin complex

DESTRUCTION OF MICROORGANISMS

Many microorganisms including some pathogenic species, are killed by fresh serum It appears to be reasonable to postulate that these substances sometimes function in vivo as they do in vitro and that they constitute *important host defense factors*

Killing of gram positive microorganisms by serum is attributed to the action of beta lysin a bactericidal system which has been discussed above In brief, the system consists of two heat stable components (I and II) of unknown nature which bear no resemblance to antibody and complement Sera from healthy human beings contain relatively low levels of beta lysin activity due to a deficiency in component I In contrast acute phase sera are very active owing to a rapid rise in concentration of component I during the early stages of an infection (Myrvik and Leake 1960)

Gram positive organisms are also susceptible to the action of lysozyme This enzyme which occurs in human serum in a concentration of approximately 5 μg/ml attacks mucopeptide complexes in bacterial cell walls Some of the bacteria which are most easily lysed by lysozyme belong to the genera *Micrococcus Sarcina Staphylococcus* and *Bacillus* (Salton 1957) Thus at least part of the bactericidal activity of serum for gram positive microorganisms may be due to lysozyme

The destruction of gram negative bacteria is commonly ascribed to the action of complement and antibody Some of these organisms undergo overt lysis when treated with serum whereas others are killed without lysis If lysis occurs as in the case of *Sh dysenteria S typhosa E coli* and *V cholerae* it results from primary damage to the cell wall not to the plasma membrane Lysis is preceded by conversion of these

organisms to spheroplasts According to Muschel and Treffers (1956) destruction of one bacterial cell of *S typhosa* requires 700 to 860 molecules of antibody and 1 5 $\times 10^7$ molecules of complement the latter being estimated on the basis of complement nitrogen values and an assumed molecular weight of complement of 160 000

While lysozyme cannot lyse gram negative bacteria as long as their cell wall is intact it definitely can do so in the presence of EDTA or after certain pretreatments of the organisms (Salton 1957 Repaske 1958) The cell wall has been demonstrated to contain the mucopeptide substrate of lysozyme (Mandelstam 1961) however the molecular arrangement of the wall is such that the enzyme cannot gain access to it The mucopeptide complexes are probably covered by lipid or lipoproteins and they become accessible only after the organization of the lipid moiety has been impaired Since complement seems to affect the lipid moiety in cell walls treatment with complement should render gram negative cells susceptible to lysozyme Consequently lysozyme should enhance the bactericidal effect of antibody and complement although *per se* it is not bactericidal for gram negative bacteria Indeed enhancement of the bactericidal action of antibody and complement by lysozyme has been observed (Wardlaw 1962 Muschel 1963)

Promotion of Phagocytosis

Serum promotes phagocytosis by interacting with the particle to be ingested (Wright and Douglas 1904) and not by supporting the function of the phagocytic cell as Metchnikoff believed The action of serum on microbes and other objects results in an alteration of their surface properties thereby facilitating their attachment to phagocytes Factors responsible for phagocytosis promoting activity of serum are called opsonins A number of different opsonins have been distinguished and include antibody complement and heat labile factors of unknown nature Whether one or the other opsonin comes into play in a given situation depends largely on the nature of the surface constituents of the object to be ingested

In vitro Studies Microorganisms In relation to opsonin requirement for phagocytosis bacteria can be divided into 3 general classes some which require no serum for ingestion others which are engulfed when either heat labile opsonin or antibody is present and still others representing encapsulated strains which are ingested only in the presence of specific antibody (Hirsch and Strauss 1964) The first class is exemplified by group A streptococcus devoid of hyaluronic acid capsule and M protein the second by *Staphylococcus albus* and the third by *Klebsiella C* Obviously immune antibody exerts in most instances an adequate opsonic effect on microorganisms so that additional heat labile components are rarely needed However in the absence of specific antibody phagocytosis depends heavily on heat labile opsonins Therefore it is likely that these latter factors assume a special significance in vivo when immune antibodies to invading microorganisms have not yet reached effective serum concentrations

Heat labile opsonin shares its most characteristic feature thermolability with both natural antibody and complement However according to Hirsch and Strauss (1964) it is not identical with either These authors studied heat labile opsonin in rabbit serum and found that it differs from natural antibody in that it lacks specificity for bacterial antigens does not function at 0 C is not a γ globulin and is inactivated by dilute ammonia or hydrazine While hydrazine sensitivity and temperature dependence of the opsonic reaction would relate heat labile opsonin to hemolytic complement complete independence of the reaction of bivalent cations seems to preclude an identity However the possibility exists that the material is related to at least part of the complement system namely to one of the two hydrazine labile components Indeed there is evidence suggesting that β_{1C} globulin constitutes a factor of the heat labile opsonin system in human serum Regarding the mode of action of heat labile opsonin on bacteria information is scarce except for the important fact that it combines firmly with the bacterial cell surface (Hirsch 1964) Serum opsonins act by combining with constituents of the bacterial surface thereby neutralizing their

only antitail antibody is able to neutralize the phage The surface of the phage tail is visualized to contain a repeating pattern of antigenic structures which enables it to accommodate a large number of antibody molecules However neutralization of a phage particle is effected by only a very few antibody molecules in fact a single molecule may suffice This is inferred from kinetic studies which indicate that the rate limiting step is the reaction of 1 antibody molecule with 1 virus particle (Cann and Clark 1956) Various aspects of the problem of virus neutralization by antibody have been discussed by de St Groth (1962) and by Talmage and Cann (1961)

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rat erythrocytes is strikingly reduced (Spiegelberg *et al* 1963) The rate of clearance becomes normal even in these decomplemented mice if the bacteria or the red cells are treated with fresh mouse serum in vitro prior to injection If they are incubated with heat inactivated serum instead restoration of clearance is not observed Since opsonization of both rat erythrocytes and *E coli* with fresh mouse serum is completely prevented by EDTA it may be concluded that the opsonizing factor is related to hemolytic complement

NON-COMPLEMENT BINDING ANTIBODY The participation of complement in phagocytosis in vivo was corroborated further in studies employing antibody rendered incapable of binding complement Pepsin digestion of antibody produces a bivalent 5S antibody fragment which is still capable of precipitation and agglutination of antigen but has lost most of its ability to fix complement Erythrocytes coated with such digested antibody were eliminated from the blood of mice as slowly as untreated erythrocytes (Spiegelberg *et al* 1963) The antibody used had an s rate of 7S and on treatment with mercaptoethanol became nonhemolytic although its s rate and valency were unchanged Evidently it had lost the intramolecular organization which is a prerequisite for complement fixation However it had also lost its opsonic function (Miescher *et al* 1963) This is good evidence for an opsonin function of complement or parts thereof in vivo These studies were performed in mice the results are not necessarily representative for all other mammalian species In the human for instance certain erythrocytes coated with non-complement fixing antibody are cleared rapidly (Mollison 1962)

Immune Adherence Nelson (1963) has furnished evidence that phagocytosis of microorganisms in vitro and in vivo might be aided substantially by their adherence to erythrocytes Human and monkey red cells possess receptors on their surface allowing the attachment of particles which are coated with antibody and complement The nature of the cellular receptor site is unknown but the component of complement responsible for the adherence phenomenon has been determined and is according to Nelson identical with β_{1C} globulin Thus a complement treated erythrocyte which has reached the state EAC 1a 4 2a 3 will give rise to a positive immune adherence phenomenon on addition of primate cells This can be observed under the microscope or can be recognized macroscopically by overt agglutination Pertinent in this connection is the demonstration by Nelson that phagocytosis of bacteria in vitro is considerably enhanced in the presence of primate erythrocytes Apparently the phagocytic cell can get hold of a small particle more easily when it adheres to a large surface The erythrocytes are not ingested under these conditions

Immunoconglutinin. This is an autoantibody to fixed complement which is produced in man and animals in response to the invasion of the body by microorganisms (Coombs *et al* 1961) It appears to be a typical γ globulin with a sedimentation rate of 7S and 19S The specificity of immunoconglutinin is directed against chemical groups of complement which are hidden in the native molecules but are revealed during the interaction of complement with antigen antibody complexes Lachmann (1962) found that cells in the state EAC 1a 4 2a are not conglutinated i e agglutinated by immunoconglutinin However treatment of this intermediate complex with isolated β_{1C} globulin rendered it highly susceptible to conglutination Thus in all probability the antigenic determinants which are reactive with immunoconglutinin are derived from β_{1C} globulin That this protein undergoes characteristic physicochemical changes during the complement reaction has been discussed above

Coombs *et al* (1961) have accumulated evidence indicating that immunoconglutinin has an effect on resistance to bacterial infections In the mouse the conglutinin level can be raised readily by the injection of either live or killed bacteria Mice with immunoconglutinin in their circulation clear bacteria from the blood more rapidly than control mice furthermore bacterial counts of liver and spleen show enhanced intracellular killing

Attraction of Leukocytes by Serum Factors In concluding the discussion of phago

antiphagocytic effect, which otherwise would prevent attachment to phagocytes

In the case of some group A streptococci two distinct antiphagocytic surface materials can be distinguished M protein and hyaluronic acid To render these microbes susceptible to engulfment by phagocytes 2 different opsonins are required specific antibody to M protein and a heat labile factor The latter differs from the heat labile opsonin discussed above, since it occurs in human but not in rabbit serum The factor has been shown to counteract the antiphagocytic effect of the hyaluronic acid capsule (Hirsch and Church 1960) it does not seem to be a component of complement since serum can be depleted of it without affecting any of the classic complement components (Stollerman *et al*, 1963)

According to Jenkin (1963) serum opsonins may have important functions beyond the promotion of phagocytosis and also play a role in determining the fate of ingested bacteria In the absence of serum opsonins certain bacteria survived and multiplied intracellularly whereas they were killed in the phagocytes when previously exposed to serum

ERYTHROCYTES The opsonic requirements for the ingestion of red cells apparently differ from those of bacteria This might be expected in view of their rather different surface properties Antibody alone is not a sufficient opsonin for red cells a heat labile material is necessary in addition to antibody Nelson (1962) identified the heat labile material as part of the hemolytic complement system He established the requirements of the 1st the 2nd and the 4th component and of C 3c which is analogous to β_{1C}-globulin Thus the complement reaction need not go to completion for the production of an opsonic effect The critical condition appears to be the presence of β_{1C} globulin on the cell surface Antiserum to β_{1E} globulin and to β_{1C}-globulin have been shown to inhibit the ingestion of red cells (Gerling Petersen and Pondman 1962)

In Vivo Studies The nature of opsonic factors operative in vivo is difficult to determine, since the experimental conditions are not readily controlled In spite of intrinsic difficulties the problem has been tackled by several investigators, and interesting results have been obtained In vivo phagocytosis of gram positive and gram negative organisms and of colloidal particles like carbon is dependent on serum opsonins These opsonins are thought to be identical with antibody and complement and it is felt that there exists a reciprocal relationship between the quantities of the two substances required for effective opsonization

DEPLETION OF OPSONIN The critical role of serum factors for the clearance of bacteria and colloids in vivo has been demonstrated convincingly through studies of opsonin depletion Injection of a large dose of carbon into a mouse causes a marked reduction in the rate of clearance of a second dose given a short time after the first If bacteria are injected instead of carbon the rate of clearance is similarly low Initially the phenomenon was interpreted to indicate saturation of the phagocytic cells by the first carbon dose However Jenkin and Rowley (1961) showed that it is not the capacity of the phagocytes which is exhausted but the amount of available opsonin in the circulation Opsonization of the bacteria or the carbon with fresh serum in vitro resulted in their prompt clearance from the blood on injection into opsonin-depleted animals

DECOMPLEMENTATION The role of complement or other heat labile factors in opsonization has been particularly difficult to establish One approach to the problem involves the use of avian antibody as sensitizer Avian antibodies bind mammalian complement rather poorly Bacteria sensitized with this antibody and then injected into animals should be cleared less efficiently than bacteria coated with mammalian antibody provided that complement has an opsonic function in vivo That this is so has been demonstrated by Benacerraf and Miescher (1960) A more direct approach involves decomplementation of the animals which are going to be subjected to phagocytosis studies Injection of heat aggregated γ globulin into the peritoneal cavity of mice is followed by a drop in serum complement activity to less than 5 per cent of the original value within 2 hours (Biozzi and Stiffel 1962) In such animals the rate of clearance of antibody coated *E coli* and of sensitized

antigen antibody precipitates Nature *199* 819

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cytosis promoting activity of serum mention should be made of still another important aspect A prerequisite for engulfment of harmful microbes is their recognition by phagocytic cells It is well known that mammalian leukocytes migrate actively toward clumps of bacteria and immune precipitates Apparently, certain diffusable substances are produced in the course of immune reactions which are instrumental in attracting these cells Although the nature of this material is still enigmatic some clues have been obtained recently Boyden (1962) found that antigen antibody complexes *per se* exert no chemotactic effect on rabbit polymorphonuclear leukocytes in vitro however on interaction of these complexes at 37° C with fresh serum the active principle is generated It is heat stable and nondialysable Since it is produced only from fresh and not from previously heated serum it might be derived from complement

In vivo experiments on rats also suggest that complement fixation is essential for the attraction and the accumulation of neutrophils in the tissue (Ward and Cochrane 1964) In normal animals deposition of immune complexes in the vessel walls leads to the infiltration of neutrophils and the development of Arthus vasculitis In animals decomplemented with aggregated γ globulin or antibody against rat β_{1C} globulin an Arthus reaction could not be elicited Polymorphonuclear leukocytes were not attracted to the deposited antigen antibody complexes Inhibition of the reaction was correlated with lack of complement binding in the tissue

IMPAIRMENT OF HOST RESISTANCE DUE TO HEREDITARY DEFECTS

Congenital Agammaglobulinemia in Man

The disease was first described by Bruton (1952) and since has been investigated extensively (Good and Zak 1956 Good *et al* 1962) Afflicted patients exhibit gross deficiency in plasma cells and absence of γ globulin from serum Immunoelectrophoresis has disclosed lack of all 3 major γ globulins 7S γ globulin 19S γ globulin and β_A globulin (Gitlin *et al* 1956) Isolated deficiency in 19S γ globulin and β_A globulin in the presence of a normal concentration of 7S γ globulin has also been observed (Gideon *et al* 1957) Family studies suggest that the disease is due to an inborn error of metabolism transmitted as a sex linked recessive trait

The main clinical feature of the disease is an increased susceptibility to bacterial infections These are primarily due to *Staph aureus* pneumococci, streptococci meningococci and *H influenza* These patients die of infection if not protected by frequent administration of pooled γ globulin

Congenital Complement Deficiency in Rabbits

In 1961 Rother and Rother reported the occurrence of a recessively inherited complement defect in rabbits Analysis of the serum revealed the absence of one of the factors of the classic third component of hemolytic complement The identity of the deficient factor has not been determined but it must be either C 3 C 5 or C′6 since incubation of the serum with sensitized cells yields a rapidly decaving EAC′1a 4,2a complex Immunoelectrophoresis and starch gel electrophoresis indicate the lack of a β globulin which has not been characterized in detail The serum of deficient animals is devoid of bactericidal activity although antibody production appears to be unimpaired An Arthus reaction cannot be elicited unless exogenous complement is supplied Despite continuous efforts to increase the number of these interesting animals only a few are alive at present because of the high mortality rate during the first 2 weeks after birth (Rother personal communication)

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10

Phagocytic Cells

INTRODUCTION AND HISTORICAL ASPECTS

It is difficult or impossible to identify the discoverer of phagocytic cells or the precise date of their discovery In the mid 1800 s mammalian tissues and fluids were studied extensively under the microscope among the types of cells recognized were some which exhibited locomotion and showed a tendency to engulf foreign particles in their environment For many years these ameboid cells were thought to function as scavengers serving to carry away debris introduced into tissues It was also widely held that they accounted for spreading infectious diseases by ingesting and transporting microbes

Precise staining procedures introduced by Ehrlich enabled the classification of ameboid blood cells into several types including neutrophils eosinophils and monocytes Studies by von Recklinghausen Aschoff and others on the localization of vital dyes injected into animals led to the recognition of two groups of large ameboid cells in tissues one freely wandering and the other apparently fixed to the inner linings of small vessels in organs such as the liver the spleen and lymph nodes

Elie Metchnikoff a zoologist by profession began his classic studies on these ameboid cells of animal tissues in 1882 It was he who gave them the name phagocyte derived from the Greek for eating cell Metchnikoff s great contribution for which he shared the Nobel Prize with Ehrlich in 1908 was the discovery that phagocytes were not mere scavengers but rather constituted one of the principle agencies of host resistance against microbial invaders (Metchnikoff 1892 1905) Metchnikoff observed closely a variety of experimental infectious diseases ranging from inoculation of a yeast like microorganism into daphnia a tiny crustacean to the introduction of anthrax bacilli into mammals and concluded that the outcome—disease or no disease—was determined largely by the interaction between the microbes and the phagocytic cells of the host As a rule whenever phagocytes accumulated about the injected microorganisms and engulfed them no disease followed whereas inoculation of microbes that resisted the phagocytic attack resulted in fulminating infectious disease

Metchnikoff's concept of the role of phagocytes in host resistance to sepsis has been amply confirmed In this chapter we shall review the current state of knowledge of the biology and the functions of mammalian phagocytic cells

MORPHOLOGY OF VARIOUS TYPES OF PHAGOCYTIC CELLS

Phagocytic cells have been classified into various types on the basis of their morphology and staining reactions on exposure to mixtures of acid and basic dyes

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eral groups monocytes and macrophages Monocytes are slightly larger than granulo cytes and in stained preparations show an oval or horseshoe shaped nucleus and abundant basophilic cytoplasm with fine azuro philic granulations Macrophages vary con siderably in morphology depending on their tissue localization and physiologic status These morphologic differences have been studied extensively (Maximow 1932) and have led to a multitude of names for the various types of cells—histiocyte clasmato cyte Kupffer cell alveolar macrophage or dust cell and so on These cells share general morphologic features such as large size round or oval nucleus and abundant baso philic cytoplasm with variable granularity Differences between these macrophages are brought out on supravital staining or on study of their ultrastructure

Living phagocytic cells observed under phase contrast optics are shown in Figure 1 The migrating human neutrophil and eosinophil both show a ruffled border or pseudopod at the leading edge and con striction into a tail like structure at the rear often with thin cytoplasmic strands extending from the trailing edge Granules in the cyto plasm are tiny and indistinct in the neutro phil but are well visualized in the eosinophil Nuclear lobes are homogeneous or slightly mottled in appearance A rabbit alveolar macrophage is shown in the lower portion of Figure 1 Among the distinguishing fea tures are the large size the ruffled membrane with microvillous projections and the large number and variety of cytoplasmic formed elements Small granules of macrophages commonly are gathered in the perinuclear region forming a rosette pattern about the centrosphere region Various types of large granular elements are located in the periph eral cytoplasm

Electron microscopic features of neutro phils eosinophils monocytes and macro phages are illustrated in Figures 2 and 3 Noteworthy aspects of the neutrophil in clude peripheral condensation of dense ma terial in the nuclear lobes the large num ber of cytoplasmic granules appearing as electron dense homogeneous bodies sur rounded by a membrane and the relative scarcity of other cytoplasmic organelles Eosinophils resemble neutrophils in general their granules are larger in most species and a dense crystalloid structure is commonly seen in the otherwise homogeneous granule matrix The monocyte shows only a few dense bodies or granules in the cytoplasm mitochondria Golgi apparatus and endo plasmic reticulum may be abundant The rabbit alveolar macrophage shown in Figure 10 3 has in its cytoplasm a great number and variety of dense bodies granules and vesicular structures

THE LIFE HISTORY OF PHAGO CYTIC CELLS ORIGIN DISTRIBUTION AND FATE

Neutrophils and eosinophils are both pro duced in the bone marrow from stem cells passing through well known stages of ma turation Myeloblast ⟶ promyelocyte ⟶ myelocyte ⟶ metamyelocyte ⟶ juvenile or stab form ⟶ mature granulo cyte (reviewed by Wintrobe 1962) Early forms are rich in endoplasmic reticulum and show cytoplasmic basophilia These morpho logic signs of protein synthesis disappear as maturation progresses and cytoplasmic gran ules are formed Division of the nucleus into lobes occurs late in neutrophil differ entiation

Use of modern radioactive technics has enabled reasonably precise determination of the distribution and the turnover of neutro phils (Osgood 1954 Bryant and Kelly 1958 Athens *et al* 1961 Brecher *et al* 1962a) Maturation in the marrow from stem cell to fully developed granulocyte re quires 2 to 3 days In a healthy man ap proximately 25 billion neutrophils are cir culating in the bloodstream at a given time A similar number of these cells are margin ated on vessel walls For every circulating neutrophil approximately 100 mature cells are held in the bone marrow reserve pool Neutrophils remain in the bloodstream for 6 to 12 hours and then pass into the tissues by traversing capillary walls Once in tissues they never return to the circulation The life span of neutrophils in tissues is not known precisely but they are end cells incapable of division and probably die or pass into the

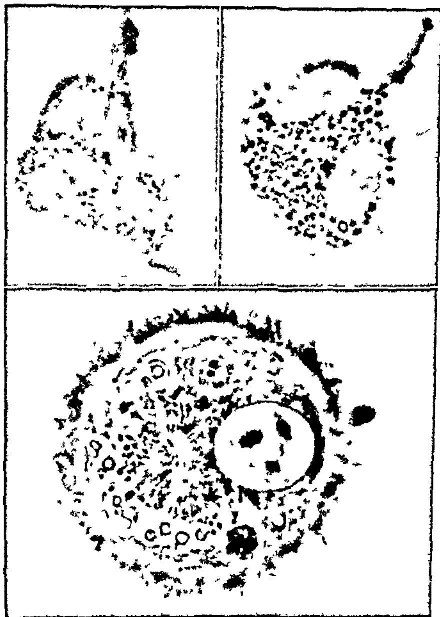


FIG 1 Phase contrast appearance of (*top left*) a human neutrophil (*top right*) a human eosinophil and (*bottom*) a rabbit alveolar macrophage in thin glass coverslip preparations × 2 000 The neutrophil and the eosinophil show a ruffled membrane at the leading edge and a tail like structure at the rear Nuclear lobes and cytoplasmic granules are evident The alveolar macrophage shows myriads of microvillous surface projections Nucleus and nucleoli are well visualized Cytoplasmic structures may be divided into 3 zones (1) a collection of small dense granules arranged in a radiating pattern about the centrosphere region and adjacent to the nucleus (2) a peripheral zone containing large granules and inclusion bodies of varied size and shape and (3) perinuclear small rod shaped structures

Polymorphonuclear phagocytes of 2 types are recognized neutrophils and eosinophils Neutrophils have a nucleus divided into several oval or sausage shaped lobes connected one to another by thin strands of nuclear material Their cytoplasm shows little or no basophilia and contains numerous (50 to 200) tiny granules which do not show a preference for either the acid or the basic stain giving rise to the names granulocyte and neutrophil In some species such as rabbit and guinea pig the cytoplasmic granules are acidophilic thus requiring the term heterophil or pseudoeosinophil For the sake of clarity and convenience in this discussion we shall employ the term neutrophil for all cells of this class

Eosinophil leukocytes commonly show a nucleus divided into 2 lobes shaped like tear drops Their cytoplasm is weakly basophilic Granules in eosinophil cytoplasm have as the name implies a strong affinity for acid dyes The size and the number of eosinophil granules varies markedly from species to species

Mononuclear phagocytes fall into 2 gen

outside world in respiratory or intestinal contents after a few days at most The picture then is one of production of neutrophils in large numbers with dynamic turnover and transportation to the tissues via the blood stream Little is known about mechanisms that regulate the rate of neutrophil production or the rate of release of mature neutrophils from the bone marrow into the blood stream (Gordon *et al* 1960)

Eosinophil life history has also been studied extensively and resembles closely the life history of the neutrophil (Rytomaa 1960) Marrow maturation of eosinophils requires approximately 2 days (Bryant and Kelly 1958) Eosinophils in the bloodstream pass into the tissues within a few hours For every circulating eosinophil there are approximately 200 mature cells in the marrow reserve and 500 eosinophils in tissues The tissues notably rich in eosinophils are intestines skin respiratory tract and vagina i e sites in contact with the outside world Eosinophils are probably incapable of division and survive in tissues for only a few days

Despite extensive study the life history of mononuclear phagocytic cells remains poorly understood Monocytes of the blood may be produced from stem cells in bone marrow or may arise from cell differentiation in lymph nodes or spleen In contrast with the granulocytes mature monocytes have the necessary equipment for various synthetic activities for long term survival and possibly for cell division The life span of circulating monocytes and their fate in tissues is not known

Macrophages include a variety of large mononuclear phagocytic cells distributed throughout various organs and tissues These cells derive from embryonic mesenchymal elements (Maximow 1907) As mentioned above they differ somewhat from one another in morphology but appear to be related functionally as reflected by their ability to concentrate vital dyes and their phagocytic activity (Aschoff 1924) Macrophages occur in particular abundance in liver spleen lymph nodes omentum lung and subcutaneous tissue The numbers and also the activity of macrophages in a given tissue change following various stimuli The exact mechanism underlying this cell proliferation and alteration is not clearly understood in some instances monocytes from blood may emigrate into the tissues and undergo transformation into macrophages (Ebert and Florey 1939) whereas in other situations existing tissue macrophages or primitive cells in lymph nodes or spleen may be stimulated to multiply or to alter their morphology and functional capacity

Macrophages may also transform into large sessile or epithelioid cells under the influences of such agents as tubercle bacilli These epithelioid cells together with giant multinucleated cells thought to arise from fusion or amitotic division of macrophages are prominent features of the granulomatous response seen in certain chronic infectious or allergic conditions

BIOCHEMISTRY AND GENERAL PHYSIOLOGY OF PHAGOCYTIC CELLS

Carbohydrate metabolism of neutrophils has been studied extensively in recent years (reviewed by Karnovsky 1962) These cells derive their energy from the breakdown of glucose or other simple carbohydrates and they carry reserve stores of such nutrients in their cytoplasm in the form of glycogen Even under aerobic conditions neutro

Fig 2 Low power electron micrographs (*Top*) a human neutrophil (*bottom*) a human eosinophil In both cells the nuclear lobes (N) show dense staining peripherally The very small dark dots distributed throughout the cytoplasm are glycogen deposits Neutrophil granules (G) are round oval or rod shaped with a homogeneous dense matrix and a limiting membrane visible in some instances A few of the granules appear to be partially empty Mitochondria and endoplasmic reticulum are sparse or absent The eosinophil granules (G) are large round or oval structures with a denser crystalloid inclusion within them A few mitochondria (M) and many small vesicular bodies are also seen Fixed in phosphate buffered osmic acid embedded in epon double stained with uranyl and lead ions × 17 600

FIGURE 2 (*Caption on facing page*)



FIG 4 An electron micrograph showing a polymorphonuclear leukocyte (1) crawling through a capillary wall The lumen of the blood vessel is at the top The leukocyte has extended a long pseudopod (Ps) between the endothelial cells (E) (From Marchesi V T Quart J Exp Physiol *46* 1961)

phils derive most of their energy from glycolysis not respiration These phagocytes continue to function well in the absence of oxygen or after the addition of cyanide to their environment on the other hand function is blocked by iodoacetate or other inhibitors of glycolysis Neutrophils degrade glucose by the Embden Meyerhof pathway and to some extent utilize the hexose monophosphate shunt They produce large amounts of lactic acid as a result of their glycolytic metabolism A functioning Krebs s cycle has been reported but is not considered to be a major metabolic pathway in the cell Normal neutrophils consume oxygen and possess respiratory capabilities Approximately two thirds of the oxygen uptake is cytochrome linked and presumably associated with mitochondrial activity whereas the remaining oxygen consumption is non-cytochrome linked and apparently reflects the activity of cytoplasmic oxidases

The synthetic abilities of mature neutrophils are largely unknown Although labeled precursors are incorporated into ribonucleic acid neutral lipids phospholipids and pro

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FIG 3 Low power electron micrographs (*top*) a human blood monocyte (*bottom*) a rabbit alveolar macrophage

The monocyte has a large horseshoe shaped nucleus (N) Stacks of small membranelike structures the Golgi complexes (Go) are prominent The cytoplasm also contains many mitochondria (M) a few dense granules numerous small vesicles and glycogen deposits × 17 600

The alveolar macrophage shows in its cytoplasm many large homogeneous dense granules (G) arranged in a radiating pattern about a clear zone Also seen are dense inclusion bodies (IB) many of which have an onion skin or myelin figure appearance Very small vesicular structures are numerous and endoplasmic reticulum is visible in some regions × 14 000

FIGURE 3 (*Caption on facing page*)

cussed later Phagocytosis and pinocytosis are probably similar processes differing only in the solid or the liquid state of the material engulfed and the size of the invaginated membrane pocket (Holter 1959)

THE INFLAMMATORY RESPONSE

In the sequence of events whereby phagocytic cells function in host defense against infectious diseases the first essential step or series of steps involves mechanisms for establishing contact between these cells and the invading microbes This contact is established in broad terms by one of two means (1) movement of phagocytic cells to the site of bacteria lodged in tissues or (2) movement of bacteria in the lymph or the blood circulation to the site of fixed phagocytic cells of the reticuloendothelial system in certain organs such as lymph nodes the liver and the spleen

Participation of Phagocytes in the Local Inflammatory Response to Microbes Lodged in Tissues

When microorganisms gain entry into tissues there follows a series of changes leading to inflammation with redness swelling heat and pain as the gross signs of this reaction Studies on the microscopic and the biochemical aspects of the inflammatory reaction are too extensive to be considered in general fashion here current knowledge in this field has been reviewed recently (Spector and Willoughby 1963) We shall limit our discussion to the mechanisms whereby phagocytes accumulate in regions of microbial invasion and inflammation

A regular feature of the microscopic appearance of inflamed areas of various types and duration is heavy infiltration with phagocytes Direct continuous observations of the inflammatory reaction in living tissue of the rabbit ear chamber (Ebert and Florey 1939 Allison *et al* 1955) has established clearly that most of the phagocytic cells in these sites come from the bloodstream they pass through the walls of capillaries and move to the infected or the injured area

In the normal capillary vessel red cells usually stream through the central zone whereas leukocytes are seen to tumble along in a relatively clear layer of plasma at the periphery Very soon after injury or the introduction of microbes the endothelial lining of small vessels in the vicinity becomes sticky The exact nature of this change is not known but in any event leukocytes begin to adhere to the wall forming a sort of a pavement lining the vessel (Florey 1962)

White cells adhering to the endothelium crawl about and soon pass through the vessel wall to reach the tissues The precise mechanism by which white cells accomplish this remarkable feat of traversing the apparently intact capillary wall has been studied recently by electron microscopy In experimental pancreatitis endothelial cells seem to send out processes and actually engulf the leukocytes (Williamson and Grisham 1961) Studies have also been done by Marchesi and Florey (1960) and by Marchesi (1961) on phagocytes emigrating through capillaries of the rat mesentery their pictures one of which is reproduced in Figure 4 clearly show the phagocyte sending out a pseudopod to penetrate the junction between endothelial cells This junction ordinarily tightly closed is thus opened whether this opening is accomplished by mechanical forces alone or also involves the secretion of enzymes to dissolve the cement substance remains to be determined

Following their emigration from blood vessels phagocytes congregate in the injured and infected area This accumulation may be based either on chemotaxis i e specific attraction and directed locomotion toward the invading microbes or on random locomotion of phagocytes and immobilization of those cells wandering into the challenge area

During the early stages of an inflammatory response to bacteria neutrophils predominate only a few eosinophils and mononuclear phagocytes being in evidence In prolonged inflammatory reactions such as those due to chronic infection macrophages often constitute a large proportion of the cells present Formerly it was held that neutrophils were specifically attracted to the acutely inflamed area and that macrophages moved in later perhaps as a result of neutrophil degeneration or biochemical changes

tem the net synthesis of these compounds has not been demonstrated (reviewed by Karnovsky, 1962) Enzymes responsible for the synthesis of glycogen are apparently present

Essentially nothing is known concerning metabolic activities of eosinophilic leukocytes

Knowledge concerning the metabolism of monocytes and macrophages is sparse, but it is believed that metabolic pathways similar to those in neutrophils are operative Glucose utilization and oxygen consumption of macrophages are somewhat higher than in neutrophils (Stahelin *et al* 1956) Recent evidence suggests that various types of mononuclear phagocytes may differ in their metabolism For example alveolar macrophages show greater respiratory activity than do the other phagocytic cells and seem to be dependent on an aerobic environment for their function (Oren *et al* 1963)

All 3 types of phagocytic cells contain a number of hydrolytic enzymes which are thought to be concerned with the degradation of ingested foreign material These enzymes include peroxidase alkaline and acid phosphatase acid ribonuclease acid deoxyribonuclease lipases proteinases beta glucuronidase and lysozyme Recent studies on neutrophils (Cohn and Hirsch 1960) eosinophils (Archer and Hirsch 1963a) and macrophages (Cohn and Wiener 1963a) show that these digestive enzymes are contained in or firmly attached to cytoplasmic granules of the phagocytic cells Therefore these granules can be considered to be a form of the *lysosome* originally described by de Duve in rat liver homogenates (de Duve 1955 Novikoff 1961)

The physiologic functions of granulocytes and macrophages center about surface phenomena resulting in locomotion phagocytosis and pinocytosis

Neutrophils and eosinophils show active ameboid movement when placed in warm chambers Locomotion can be quite rapid ranging up to 40 microns per minute (cited by Dittrich 1962) Monocytes and macrophages move about slowly or not at all under in vitro conditions but do show undulating movements of their membranes After prolonged culture on glass surfaces they commonly assume a stellate shape Mononuclear phagocytes observed in tissues move slowly

All 3 types of phagocytic cells move toward or away from certain objects or substances in their environment a phenomenon termed chemotaxis Chemotaxis of leukocytes has been studied for nearly a century Many of the studies on this phenomenon have led to fallacious conclusions based on technical artifacts (Harris 1954 1959), but it is true that once within range (usually 100 microns or less) of suitably attractive objects phagocytic cells of all types abandon their random locomotion and move in a straight line toward the object The precise mechanism involved in this sensory response of phagocytic cells remains unknown (McCutcheon 1955) Most authorities have proposed the existence of a concentration gradient of chemical nature about the particle this gradient being based either on the liberation of some substance from the particle or on adsorption onto the particle of a component of the micro-environment Also possible but unsupported by experimental evidence is a mechanism whereby a single leukocyte first encounters a particle by chance and then as a result of this encounter liberates a chemical messenger to call other phagocytes to the area Chemotaxis to bacteria such as staphylococci apparently operates in the absence of serum (cited by Florey 1962) On the other hand recent studies by Boyden (1962) indicate that chemotaxis of antigen antibody precipitates requires the presence of fresh serum The precipitates are not themselves chemotactic but rather interact with heat labile serum factors to produce a heat stable substance which attracts white cells

Pinocytosis first described by Lewis (1931) in cultures of macrophages consists of tiny invaginations of undulating surface membranes to form long thin channels extending into the cell these pinch off resulting in the formation of cytoplasmic vesicles containing fluid from the environment (Chapman Andresen 1962) Mammalian phagocytes apparently employ pinocytosis for the bulk transport of both small and large molecular weight solutes The analogous process by which solid particles are taken into the cell phagocytosis will be dis

the ingestion process and the role of leukocyte metabolism in particle uptake Under conditions in which fully susceptible bacteria are presented to phagocytes in an optimal environment it is possible to block the ingestion process by introducing certain metabolic inhibitors Studies with polymorphonuclear leukocytes clearly indicate that compounds which block the glycolytic pathway also destroyed the capacity of the cell to carry out particle ingestion (Sbarra and Karnovsky 1959 Cohn and Morse 1960a) In contrast phagocytosis is not prevented by factors which depress respiration such as an anaerobic environment cyanide or antimycin A Similar metabolic requirements for phagocytosis have also been found for peritoneal macrophages of various animals (Oren *et al* 1963) However the alveolar macrophage appears to depend on both the respiratory and the glycolytic pathways for its function

The susceptibility of bacteria to phagocytosis varies widely depending on the physicochemical nature of their surface which apparently determines whether or not fixation to phagocyte membrane can occur From this point of view microbes can be divided into 3 groups (1) a group of avirulent rough strains exemplified by rough pneumococci and some streptococci possessing no M protein or hyaluronic acid capsule whose surface apparently requires no modification in order for attachment to phagocytes to occur (2) strains such as *Staphylococcus albus* or some coliforms which are not taken in unless they have been coated either with heat labile serum factors or with antibody and (3) potentially virulent encapsulated microbes whose surface enables them to ward off phagocytosis under many conditions unless this surface is altered by reaction with specific antibody (Hirsch and Strauss 1964) The nature of the antiphagocytic surface material may be carbohydrate protein or both as illustrated by the polysaccharide capsule of pneumococci the M protein and hyaluronic acid of streptococci the polyglutamic acid capsule of anthrax bacilli or the surface factor of some strains of *Staphylococcus aureus* (see chapters on these microbes in this volume) Thus clearly established is the prime importance in phagocytosis of serum factors of antibody and/or complementlike nature collectively known as opsonins Efficient operation of host resistance in this regard requires the combined action of both humoral and cellular agencies The role of opsonins in promoting phagocytosis has been demonstrated in vitro under various conditions and also in the clearing of bacteria from tissues or the bloodstream of living animals (reviewed by Rowley 1962)

A variety of other environmental factors influence the kinetics of phagocytosis These include salt concentration pH divalent cations and temperature (Mudd *et al* 1934 Chernew and Braude 1962) In most instances the changes in the medium required to impair phagocytic function significantly are drastic and far removed from physiologic conditions Phagocytosis proceeds quite well over a rather wide range of hydrogen ion concentration say pH 6 to 8 A variety of reagents which chelate divalent cations block phagocytosis these cations are apparently necessary for normal cell function and perhaps also play a role in attachment of microbes to cell membrane Salt concentrations of the medium somewhat lower than the physiologic level actually increase the rate of phagocytosis In contrast when the ionic strength is increased to approximately twice that of physiologic solutions particle uptake is blocked

In addition to chemical composition the physical nature of the environment also plays a prominent role in phagocytosis This is illustrated best by the process of surface phagocytosis as described by Wood and associates (1951) These investigators found that leukocytes in a structured environment sometimes were able to engulf encapsulated bacteria in the absence of antibody in contrast with the lack of ingestion in glass systems in vitro Direct observation of this process reveals that the phagocytes trap or cover the microorganisms on suitably rough surfaces and proced to engulf them A similar process called intercellular phagocytosis occurs in dense suspensions of leukocytes in which surrounding cells serve as the trapping surfaces

Another type of reaction which bears some resemblance to surface phagocytosis is the

associated with the evolution of the reaction More precise quantitation of phagocytes in inflammation (Paz and Spector, 1962) has revealed that all types of phagocytes move into the area initially, the macrophage pre dominance later on does not necessarily re flect specific attraction of these cells but rather may be based on death and removal of the short lived polymorphonuclear leuko-cytes in the area and at the same time survival and fixation of macrophages

Some inflammatory reactions due to aller gies or parasitic infestations show eosinophils as the predominant phagocytic cell Antigen antibody complexes may be responsible for the accumulation of eosinophils at these sites (Litt 1961, 1962) Most immune precipi tates attract all types of phagocytic cells but apparently in certain situations, not yet precisely defined specific eosinophilotatic conditions arise

Clearance of Microbes From the Blood or Lymphatic Circulations by Fixed Mononuclear Phago cytes of the Reticuloendo thelial System

In the course of the inflammatory reaction permeability of capillaries and small venules to blood fluids is increased The resulting local edema of tissues leads to distention of the lymphatic channels and an increase in lymph flow Microbes are frequently caught up in this lymph drainage and transported to the regional lymph nodes where they en counter fixed macrophages arranged in a sort of a filtering system Bacteria may be come lodged in the lymph vessel or nodes before they are taken up by fixed macro phages, and cause inflammation—lymphan gitis and lmphadenitis—with an attendant influx of blood phagocytes of all types from adjacent capillaries

Microbes caught in the lymph drainage may escape engulfment by the fixed macro phages in lymph nodes and be carried eventually to the blood In other instances microbes gain entry directly into the blood stream for example as a result of wounds or in cases of inflammation accompanied by necrosis Clearance of bacteria and other particles from blood has been studied exten sively (Halpern *et al*, 1953 Rowley 1962) Removal of microbes from blood is accom plished primarily by phagocytic activity of fixed macrophages in sinusoids of certain organs such as the liver and the spleen Even though they are present in large numbers circulating phagocytes seem to play a rela tively minor role in most instances prob ably because opportunity for contact is limited betwen leukocytes and microbes, both of which are carried along passively in the bloodstream

PHAGOCYTOSIS AND THE ENSUING *INTRACELLULAR EVENTS*

Phagocytic cells play their role in host resistance to sepsis by ingesting and inacti vating microorganisms This function can be separated into 2 stages which have different determinants The first step is the actual engulfment process in which the phagocyte surrounds the microbe by means of pseu dopods and transfers it to an intracytoplasmic locus The second stage comprises the variety of intracellular events both morphologic and biochemical which often result in destruc tion of the microorganism

Extensive discussion of general aspects of the act of phagocytosis and its consequences can be found in the reviews of Mudd *et al* (1934) Suter (1956) and Karnovsky (1962)

Determinants of the Rate of Engulfment of Microbes by Phagocytic Cells

Assuming that contact between phagocyte and microbe has been established various factors determine whether or not the microbe will be engulfed These factors include the metabolism of the phagocyte the nature of the bacterial surface the chemical com position of the extracellular medium and the physical nature of the environment For the sake of simplicity these will be discussed separately although it is obvious that all interact to produce a more complicated pic ture under natural conditions

Originally phagocytosis was thought to be a passive physical process dependent only on the surface tension of the cell and of the particle (Fenn 1921) More recent observations establish the active nature of

ment occurring in the phosphatidic acids (Elsbach 1959 Karnovsky and Wallach 1961) These changes in metabolism are unrelated to the nature of the ingested particle and their mechanism and significance are not clearly understood Tentatively they may be considered to reflect the increased demand for energy associated with the phagocytic process and possibly the synthesis of new lipoprotein membranes

During or shortly after the phagocytic process a most striking morphologic event takes place This is the degranulation phenomenon which occurs in all 3 types of phagocytic cells (Hirsch and Cohn 1960 Archer and Hirsch 1963b Cohn and Wiener 1963b) Intimate morphologic features of the degranulation reaction have been studied in polymorphonuclear leukocytes (Hirsch 1962) Granules lyse only when they come into contact with the wall of the phagocytic pouch or with clear zones resulting from prior granule rupture The upper part of Figure 6 shows on the right a neutrophil degranulated after ingesting many bacilli and for comparison on the left a normal neutrophil with numerous granules The rapidity and the detailed morphology of lysis of individual granules in a chicken neutrophil ingesting 2 yeast bodies is shown in the lower portion of Figure 6 Lysis of an individual granule is a very rapid almost explosive event During degranulation hydrolytic enzymes and antibacterial substances in the granules are liberated in soluble form (Cohn and Hirsch 1960b) and probably are transferred directly into the phagocytic vacuole Electron micro

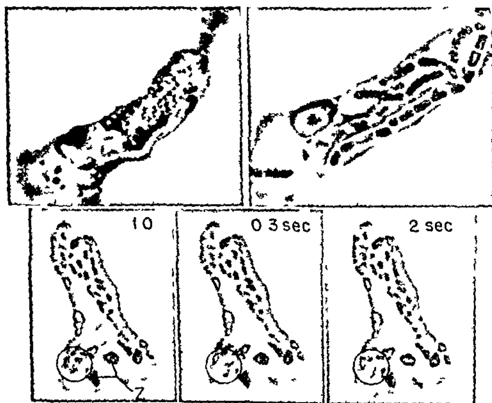


FIG 6 Degranulation which accompanies phagocytosis (*Top left*) A normal neutrophil with its full complement of granules (*Top right*) The neutrophil has engulfed numerous microbes which lie in vacuoles cytoplasmic granules have vanished × 2 000 (*Bottom*) Morphologic and temporal features of granule lysis (in the circled areas) in a chicken polymorphonuclear leukocyte during phagocytosis of yeast cell wall bodies (zymosan Z)

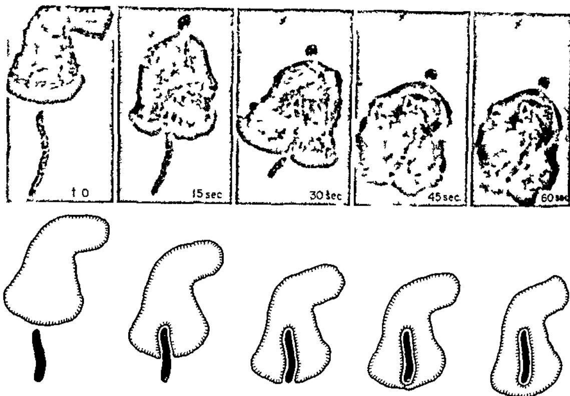


FIG 5 (*Top*) Prints from a motion picture of a human neutrophil engulfing *Bacillus megaterium* × 1000 (*Bottom*) In diagrammatic form the mechanism by which phagocytosis is accomplished i e invagination of cell membrane

immune adherence phenomenon (Nelson 1953) Certain bacteria in the presence of antibody and complement adhere to the surface of erythrocytes When this occurs leukocytes move about avidly ingesting the immobilized organisms

INTRACELLULAR EVENTS

Following the initial contact between bacterium and phagocyte pseudopod formation is stimulated and contact spreads until at the distal portion of the particle cell membranes from opposite sides meet and fuse The ingested particle is thus confined intracellularly within a pouch or phagocytic vacuole the wall of this pouch being formed by the inverted cytoplasmic membrane of the phagocyte Figure 5 shows (upper row) photomicrographs of a human neutrophil ingesting *Bacillus megaterium* The lower portion of the figure presents the phagocytic process schematically to emphasize the membrane invagination which accompanies particle uptake

During or at the completion of engulfment a variety of metabolic and morphologic events occur which have important effects on both the phagocyte and the ingested particle The majority of studies on the intracellular consequences of particle ingestion have been performed with polymorphonuclear leukocytes although a similar series of events may take place within macrophages

During the process of particle ingestion the phagocyte displays a number of changes in its metabolism The first of these to be studied was a stimulation of oxygen consumption (Baldridge and Gerard 1933) a good portion of which is not linked to the usual electron transfer scheme More recent investigations have demonstrated the stimulation of glucose consumption lactic acid production glycogenolysis and the utilization of glucose by means of the hexose monophosphate shunt (Sbarra and Karnovsky 1959 Cohn and Morse 1960a) In addition there is an increased turnover of neutral lipids and phospholipids with a major incre

microorganisms are destroyed rapidly within phagocytes These susceptible bacteria in clude among others pneumococci strepto cocci staphylococci of both albus and aureus strains klebsiella species and coliforms (Wood 1960) These microbes survive in side neutrophils for only a short time their half life being 7 to 8 minutes Whether or not all forms of macrophages are equally effective or quick in killing ingested bacteria is not known and some recent evidence sug gests that they may differ in this function (Mackaness 1960 Pavillard and Rowley 1962) Little is known about inactivation of bacteria within eosinophils

Our knowledge concerning the intracel lular bactericidal mechanisms is fragmentary A more comprehensive discussion of this field can be found in the reviews of Skarnes and Watson (1957) and Hirsch (1960a) Two bactericidal agents can be extracted from neutrophils of various animals These are lysozyme and a factor called phagocytin Lysozyme was discovered by Fleming (1922) it is a low molecular weight basic protein which acts enzymatically to degrade certain acetylaminopolysaccharides Microbes whose cell wall contains this substrate in form accessible to the enzyme are lysed on exposure to lysozyme Other microbes not susceptible to direct attack by lysozyme are killed on exposure to combinations of this substance and antibody (Amano *et al* 1954) or certain chelating agents (Repaske 1956) Phagocytin has a wide spectrum of activity against both gram positive and gram nega tive bacteria (Hirsch 1960b) It has prop erties suggestive of a basic protein (Zeya and Spitznagel 1963) but has not been iso lated in a pure form as yet Both phagocytin and lysozyme are associated with the gran ules in the normal cell and are liberated at the time of phagocytosis (Cohn and Hirsch 1960)

In contrast with the neutrophil peri toneal macrophages and blood eosinophils contain no phagocytin or lysozyme (Myrvik 1960 Hirsch 1960b Archer and Hirsch 1960a) and alveolar macrophages contain lysozyme but no phagocytin (Myrvik 1960 Cohn and Wiener 1963a) Therefore it is apparent that much remains to be learned about the bactericidal activity of phagocytic cells in particular the mechanism by which macrophages kill microbes remains a mys tery The possible antibacterial role of lactic acid (Dubos 1954 Locke and Rowley 1962) and hydrogen peroxide (Iyer *et al* 1961) produced during metabolism of phag ocytes should be mentioned Recent obser vations (Jenkin 1963) suggest that specific antibody and possibly complement may sen sitize strains of salmonella for killing within macrophages As yet no such effect has been noted for polymorphonuclear leukocytes

Although most microorganisms are rapidly inactivated within phagocytes there are several instances in which this is not the case For example the tubercle bacillus survives within both granulocytes and macrophages *Brucella abortus Pasteurella tularense* some strains of salmonella and *Listeria monocyto genes* have also been reported to survive and multiply within peritoneal macrophages of laboratory animals

Following the bactericidal event suscepti ble bacteria are gradually degraded within phagocytic cells This observation was made initially by Metchnikoff (1905) on morpho logic grounds and has been documented repeatedly by subsequent investigators In gested microorganisms soon lose their baso philia and in some instances their struc tural integrity More exact biochemical information indicates that degradation of bacterial macromolecules takes place rap idly and that in some instances the phagocyte can use these products as substrates for its own synthetic or metabolic processes (Cohn 1963) The rate and the extent of digestion of bacteria are similar within neutrophils and macrophages In the course of this intra phagocytic digestion bacterial toxins and antigens may be destroyed at least in some instances (Walsh and Smith 1951 Cohn 1962a)

Thus far our discussion has been related primarily to the influence of phagocytes on the ingested microorganism In some cases however bacterial products affect phagocytes adversely Although many bacterial products can damage phagocytic cells only a few have this effect in high dilution For example some strains of *Streptococcus pyogenes* pro duce streptolysins whose effect on phago cytes has been investigated recently (Hirsch



FIG 7 Electron micrograph of a rabbit polymorphonuclear leukocyte soon after ingestion of zymosan particles (Z) At two points indicated by the arrows the membrane surrounding the engulfed particle is seen to be continuous with membranes about cytoplasmic granules indicating that membrane fusion accounts for discharge of granule contents into the phagocytic vacuole × 32 000 (Zucker Franklin D and Hirsch J G J Exp Med 1964)

graphic studies suggest that this transfer is accomplished by fusion of the granule membrane with the inverted cell membrane surrounding the engulfed microorganism (Lockwood and Allison 1963 Zucker Franklin and Hirsch 1964) Figure 10 7 shows the discharge of the contents of the granule into the phagocytic pouch shortly after a neutrophil has ingested yeast cell wall (zymosan) particles

Studies on the fate of bacteria within phagocytic cells have been performed in vitro for the most part and with a variety of experimental systems One of the most direct of these has been described by Wilson *et al* (1957) the viability of strains of streptococci has been determined within individual cells at various times after ingestion by human neutrophil leukocytes Other techniques in which the over all fate of bacterial populations can be investigated have been described for neutrophils (Cohn and Morse 1959) and macrophages (Rowley 1960) In general the vast majority of common

Patients with diabetes mellitus seem to be unusually susceptible to certain bacterial diseases and the acidotic experimental animal has clearly lost its ability to ward off certain fungal agents Although the mechanism is not fully understood a delayed and inadequate phagocytic reaction is seen in the tissues of the challenged animal in diabetic acidosis (Sheldon and Bauer 1959)

The unusual susceptibility to bacterial invasion of the renal medulla has been noted in various clinical and experimental situations Recently it has been found that the phagocytic response to lodgement of microbes in the medulla of the kidney is considerably delayed as compared with that in the cortex or other tissues (Rocha and Fekety 1964) Furthermore when leukocytes finally emigrate into medullary tissue their phagocytic ability may be impaired by the high salt concentration likely to be present (Chernew and Braude 1962) or by the fact that ammonia produced in the kidney inactivates the 4th component of complement and destroys opsonic factors required for efficient ingestion of the bacteria (Beeson and Rowley 1959)

Functions of Fixed Phagocytic Cells in Clearing Bacteria From the Blood or the Lymph Circulation

The functioning of fixed macrophages in the blood and the lymphatic streams does not involve a long chain of events as described above for tissue clearance Bacteria are carried to the phagocytes by blood or lymphatic flow therefore variations in function involve primarily the numbers of filtering cells and the efficiency of their individual performance

Quantitative methods have been developed for measuring the rate of bloodstream clearance of carbon particles or labeled bacteria by the reticuloendothelial system This removal rate is influenced by many factors including the size of the particles and the presence of opsonins (reviewed by Benacerraf and Miescher 1960 and by Rowley 1962)

Clearance can be blocked or severely impaired by the injection of large amounts of certain materials such as bacterial lipopolysaccharide Thorotrast or colloidal sulfur In some instances such blockade may reflect inadequate function of overstuffed macrophages Some evidence also suggests that the less efficient phagocytosis under these circumstances may be based at least in part on depletion of opsonic factors following injection of large amounts of these colloid materials

Following the period of blockade the clearance rate not only returns to normal but often is for a time considerably faster than normal This stimulation of fixed phagocytic activity is accounted for by an increase in the number of these cells and also an increase in individual cell performance (see Rowley 1962) Higher than normal levels of circulating opsonic factors may also contribute to the efficiency of reticuloendothelial clearance at this time

In many instances the resistance of experimental animals to bacteria inoculated intravenously is found to be directly related to the efficiency with which the reticuloendothelial system is functioning

The Phenomenon of Acquired Cellular Immunity

Although the majority of the common pathogenic and saprophytic bacteria are handled effectively by phagocytic cells certain organisms are able to survive in the intracellular environment Furthermore with many of these microbes it can be demonstrated that serum antibody plays no important opsonic or bactericidal role However the animal previously exposed to such organisms often acquires a relative immunity to reinfection In order to explain this form of resistance the concept of *cellular immunity* has been invoked Work on this subject has been done with only a few intracellular parasites i e tubercle bacilli brucellae *Pasteurella tularense* and *Listeria monocytogenes* and generally has been limited to observations on their interaction with mononuclear phagocytes At the present time there is no evidence that neutrophils or eosinophils from immunized animals behave differently from cells of the normal animal

On reinfection tubercle bacilli do not multiply and may even be destroyed in contrast with the growth of the microbes seen

et al 1963) These hemolytic proteins kill polymorphonuclear leukocytes apparently by lysing their cytoplasmic granules and liberating digestive enzymes into the cytoplasm of the cell with ensuing autolysis Comparable studies by Woodin (1962) on the action of staphylococcal leukocidin have shown a similar effect However, the importance of these substances in determining the pathogenicity of these organisms has not been established

The complex lipopolysaccharide endotoxins of gram negative bacilli alter the activity of both mononuclear and polymorphonuclear phagocytes In low concentrations these substances enhance the rate of phagocytosis and of glycolysis whereas at higher levels they inhibit these parameters as well as the migration of cells from buffy coat explants (Cohn and Morse 1960b Rowley 1960)

FUNCTIONS OF PHAGOCYTIC CELLS

Role in Combating Microbial Invaders in the Tissues

The function of phagocytes in response to microbes introduced into tissues involves a rather lengthy chain of events production of mature polymorphonuclear leukocytes or mononuclear phagocytes from precursor cells in bone marrow or extramedullary sites delivery of adequate numbers of these cells to the blood margination and emigration from capillaries in or near the invaded tissue *locomotion and perhaps directed movement* toward the microbes phagocytosis transfer of bactericidal agents and hydrolytic enzymes from lysosomal granules to the phagocytic pouch or vacuole killing and digestive attack on the microbes and finally egestion excretion or utilization of the degraded microbial products

It should be emphasized that weakness in any link of this chain of events can result in an inadequate over all phagocytic performance and diminished resistance to infectious disease A few examples of naturally occurring or experimental abnormalities of phagocytic function and host resistance may help to make the point

Perhaps the role of neutrophils in host resistance to infectious diseases is illustrated best by the notorious susceptibility to sepsis of individuals afflicted with agranulocytosis a disorder in which neutrophil production in the marrow is blocked by toxic or allergic reactions

Following massive exposure to x irradiation, circulating neutrophils fall to low levels or even disappear and resistance to bacterial invasion is reduced markedly (Brecher *et al* 1962b Cohn 1962b Smith *et al*, 1963) The scarcity of phagocytic cells almost certainly accounts in part for susceptibility to infectious agents in this situation however other host resistance mechanisms such as anatomic barriers and antibody production are also altered following irradiation

Many forms of leukemia and blood dyscrasias are associated with enhanced susceptibility to bacterial disease Total numbers of circulating leukocytes may be higher than normal in some of these conditions but the bulk of the cells are immature These immature leukocytes are grossly deficient in their ability to emigrate from the blood stream (Rebuck *et al* 1961) and to phagocytize microorganisms (Jersild 1948)

Patients given large doses of certain adrenocortical steroid hormones are unusually prone to develop infectious diseases Latent microbial agents or avirulent species may produce sepsis in this setting Cortisone administration leads to an increase in numbers of circulating functionally normal neutrophils (Hirsch and Church 1961) *However under the influence of cortisone* the inflammatory process is severely suppressed or retarded the hormone apparently interferes with margination emigration and accumulation of phagocytic cells at the challenge site (Germuth 1956 Cohn 1962b) Furthermore suppression of antibody formation by these steroids and alterations in the local biochemical environment in tissues may contribute to the change in host resistance

Other agents such as levans (Shilo 1962) endotoxins (Cohn 1962b) and epinephrine (Miles 1956) act locally to block or retard the influx of phagocytic cells into tissues or body cavities and thus favor markedly the survival of microbes introduced into the affected area

of Arthus reaction sites (Daems and Oort 1962) thus autolytic enzymes released from the granules might conceivably cause local tissue necrosis or extension of the inflammatory process

A specific function for eosinophils in the inflammatory site has also been proposed Eosinophil granules contain in addition to hydrolytic enzymes substances capable of blocking the action of histamine (Kovacs 1950 Vercauteren and Peeters 1952) and of serotonin and bradykinin (Archer and Broome 1963) These 3 materials are responsible at least in part for increased vascular permeability in the inflamed area (Spector and Willoughby 1963) Therefore it is proposed that eosinophils play a homeostatic role serving to counteract or limit the effects of vasoactive substances

Finally to be mentioned is the role of phagocytic cells in general host responses to sepsis (see Chap 13) the best example of such a role being the release from neutrophils of a fever producing agent endogenous pyrogen (reviewed by Atkins 1960)

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following the initial challenge Studies by Lurie (1942) indicate that this inhibition of multiplication in the immunized animal is related to a property of its mononuclear phagocytes Suter (1953) found, in a tissue culture system that macrophages from immunized animals inhibit the intracellular multiplication of virulent tubercle bacilli whereas these bacteria multiply freely in cells from normal animals Sera from normal and vaccinated animals do not differ detectably in their effect on the fate of mycobacteria in this type of in vitro system Employing a slightly different technic Mackaness (1954) obtained contrary results namely that prior vaccination does not alter the rate of myco bacterial multiplication within macrophages of the rabbit It should be stressed that in none of these experiments do the immune macrophages have a lethal effect on the bacilli

Similar experiments employing strains of *Brucella abortus* have been reported by 2 groups of investigators (Pomales Lebrun and Stinebring 1957 Holland and Pickett 1958) In both cases macrophages from immunized animals inhibit the multiplica tion of virulent organisms

Perhaps the most conclusive studies on the topic of cellular immunity are those reported recently by Mackaness (1962) using a system of mouse macrophages and *Listeria monocytogenes* Peritoneal macro phages from the normal mouse allow mul tiplication of a virulent strain In contrast the infection of the animal with small num bers of viable bacteria results in a marked increase in the resistance of the peritoneal cells on the 4th day At this time 90 per cent of these cells rapidly inactivate an inoculum of listeria

The specificity of this reaction has been studied subsequently by Mackaness (1964) Employing *Brucella abortus* as an infecting agent, it was possible to demonstrate that peritoneal macrophages became resistant to an in vitro challenge with listeria Since there is no known immunologic relationship be- tween the two strains this implied that the macrophage alteration was nonspecific However little is known concerning the mechanism of this change nor is there ade quate data relative to the size of the macro- phage pool in the sensitized animal These questions are currently under investigation in a number of laboratories

Functions of Phagocytic Cells Unre lated to Their Interaction With Microbes

Of course phagocytic cells do play a role as scavengers that is to say, they engulf nonmicrobial foreign material and digest it or sequester it within their cytoplasm or transport it to the outside world in intestinal and respiratory tract secretions Phagocytes also ingest damaged tissue cells and effete erythrocytes the destruction of red blood cells being primarily an activity of macro phages in the spleen and the liver (Muir and Niven 1935)

Much work has been done concerning possible relationships between phagocytes and antibody production or destruction As mentioned earlier some antigens apparently are degraded within neutrophils to such an extent that they are no longer immunogenic On the other hand eosinophils (Speirs 1958 1963) or macrophages (Fishman 1961 Fishman and Adler 1963) are thought by many to play an essential role in preparing antigen in transporting antigen to the potential antibody producing cell or even in producing antibody directly

Antigen antibody complexes may exert harmful effects on host tissues Since phago cytic cells are capable of engulfing and di gesting immune precipitates (Sorkin and Boyden 1959 Cochrane *et al* 1959) they may well function to protect against these harmful effects Eosinophils seem to be par ticularly prone to accumulate about antigen antibody complexes (Litt 1961 1962) and do ingest these complexes (Sabesin 1963) degranulating during the process (Archer and Hirsch 1963b)

In some instances phagocytic cells in the inflammatory area may be responsible for damage to host tissue as well as to the microbial invaders For example Arthus or Shwartzman reactions are suppressed or even blocked in the neutropenic animal (Stetson and Good 1951 Humphrey 1955) Degranulation of neutrophils has been noted in electron microscopic studies

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TABLE 1 TYPES OF ALLERGIC INFLAMMATORY RESPONSES IN MAN*

	IMMEDIATE TYPE	
	WHEAL AND-ERYTHEMA†	ARTHUS-TYPE
Clinical state	Hay fever asthma Serum sickness Purpura Physical allergies Some infectious allergies Penicillin rashes	Serum sickness Erythroblastosis Purpura (drugs) Glomerulonephritis ? Rheumatic fever ? Rheumatoid arthritis ? Periarteritis nodosa ??Agranulocytosis ??Ulcerative colitis
Sensitizing materials	Pollen molds kapok danders Antibiotics a few drugs special allergens (ascaris toxoid horse serum etc) Altered tissues of the individual (physical allergies)	Soluble proteins Drugs antibiotics Polysaccharides of bacteria and parasites Erythrocytes (Rh positive) ? (unknown materials)
Antibody	Present in serum Nonprecipitable Heat labile	Present in serum Precipitable Heat stable
Transfer of sensitivity	With serum	With serum
Cytotoxicity of antigen for sensitive explanted cells	None	None

	DELAYED TYPE	
	MICROBIAL HYPERSENSITIVITIES	NONLIVING AGENTS
Clinical state	Tuberculosis tularemia brucellosis Lymphogranulomatosis smallpox mumps Histoplasmosis and other mycoses Trichinosis	Cutaneous rashes (Allergic contact type dermatitis) Homograft rejection ? Multiple sclerosis ? Thyroiditis
Sensitizing materials	Bacteria viruses fungi parasites	Poison ivy plant oils simple chemicals plastics antibiotics etc Histocompatibility antigens Altered tissues ?
Antibody	Not found in serum Not found within cells	
Transfer of sensitivity	With cells not with serum	
Cytotoxicity of antigen for explanted sensitive cells	Present	

* Adapted from Lawrence H S 1956 Am J Med 20 428-447

† Urticaria gastrointestinal disturbances angioneurotic edema and eczema although immediate type allergies may not exhibit cutaneous reactivity or presence of reagins Foodstuffs often represent the offending allergens The position of infantile eczema is undetermined

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11

The Allergic State

The term allergy designates an altered reactivity of the tissues toward particular substances as judged from previous experiences of a given individual with the same material or from the norm provided by other individuals of the same species * This definition must exclude the occasional instances of idiosyncrasy in which one encounters an inherent qualitatively abnormal reactivity toward physiologically active materials e g morphine Allergy as seen in man includes a wide variety of manifestations—serum sickness hay fever and asthma allergy to foods infantile eczema poison ivy dermatitis sensitivity to antibiotics and synthetic drugs and sensitivity to products of microbial origin such as tuberculin

The inciting substances indeed various in nature *induce the altered reactivity in the host* following penetration by ingestion absorption through mucous membranes or through skin mechanical injection or invasion of the tissues by pathogenic microbial *or viral or parasitic agents*

The allergic or hypersensitive state may be viewed as *an acquired capacity of the body to react against antigen* Reactions of hypersensitivity are then the outcome of what we may term *the immunologic apparatus* interacting with antigen in the presence of tissues or cells and *allergic inflammation is the common response*

To take the broadest example every animal that has received injections of a foreign protein is potentially an allergic individual for suitably tested it can exhibit anaphylactic shock or inflammatory responses at sites of intradermal injection and in addition a release of histamine and various physiologically active substances can be shown

Allergic reactions that are mediated by circulating antibody commence at once upon the formation of antigen antibody complexes and in this sense can be called immediate with regard to the time of inception of the reaction although a longer period may be required before the physiologic disturbance *has reached its maximum* Typical examples would be allergic rhinitis (hay fever) anaphylactic shock reactions of Arthus type serum sickness and experimental glomerulonephritis (Table 1) The types of *antibody that are concerned vary* as will be described below

Another major category of allergic reactions shows no obvious relationship to circulating antibody The time-course is such *that reactions become apparent only after* several hours and the maximal reaction is reached after 1 to 2 days or longer The term delayed type reactions has been applied to these tissue responses which appear to be attributable to special properties of the white cells probably of the lymphocytic series

* The term is used here in the original sense of von Pirquet (1906) who introduced it to cover all changes induced in the state of reactivity in consequence of contact with any living thing or inanimate substance

vary in their relative combining affinity (avidity) for their specific antigen First formed antibody is generally of low avidity binding with antigen but dissociating from it with fair ease With further antigenic stimulation antibody appears that is increasingly more avid Again while antibodies usually have more than one combining site and are able to bind antigenic molecules in a lattice work that eventually precipitates some classes of antibodies bind to only one molecule of antigen and consequently do not build up a precipitating complex these have been called univalent antibodies Certain Rh human antibodies for example attach to red cells but must be detected by agglutination with antihuman globulin in the indirect Coombs test

Consequently the antibodies operative in reactions of the immediate type of hypersensitivity all attuned in some measure to their inciting antigen fall into several classes However in anaphylaxis and the Arthus reaction we shall be dealing with precipitating antibodies except as specifically noted

ANAPHYLAXIS

Anaphylaxis in its primary sense is an acute *systemic* reaction species characteristic that is exhibited by sensitized animals shortly after reinjection of the same antigen

The word anaphylaxis was coined by Richet in 1902 as a term to contrast with prophylaxis in order to describe a state of *excessive susceptibility* discovered in dogs that he was attempting to immunize with toxic materials Subsequent observations made on guinea pigs by Theobald Smith Otto and many others established that the phenomenon resulted from antigen antibody reactions occurring in vivo For example serum from a guinea pig undergoing active anaphylactic sensitization could sensitize normal guinea pigs (passive transfer)

The symptom complex of anaphylaxis differs with the species such that the physiologic events set in motion by the antigen antibody reaction in vivo appeared to be baffling Some workers held that a soluble anaphylatoxin was set free in the blood stream others that the phenomenon primarily involved tissue cells Subsequent work has revealed that both groups of workers had been dealing with different but valid facets of anaphylactic shock

The discovery of histamine by Sir Henry Dale (1913) gave the first proof of a physiologically active chemical mediator of anaphylaxis The number of skeptics was legion not only was demonstration of its occurrence in all the various species difficult in view of dilution in the circulation and degradation by histaminase but it did not account for all phenomena

Today besides histamine 3 other pharmacologically active chemical materials are known to arise in vivo following antigen antibody combination (Table 2) Histamine and serotonin (5 hydroxytryptamine) both exist preformed in the tissues Two further materials are not preformed but arise *de novo* in consequence of the antigen antibody reaction namely SRS A (Slow Reacting Substance of Anaphylaxis) formed in the tissues and special short peptides plasma kinins that arise by enzymic cleavage of certain plasma proteins The responsible enzymes for engendering SRS A and plasma kinins are bound to fixed tissues (Table 2) and it is not unlikely that they may be held within intracellular granules termed lysosomes (Chap 10) awaiting degranulation by antigen antibody interaction at the cell surfaces

The other preformed substances acetylcholine and heparin appear to play minor roles although release of heparin from mast cells may act to mitigate certain features of shock The active products called anaphylatoxin and leukotaxine are still unknown chemically and it is possible that they may come to be identified with plasma kinins for example

A role has often been pictured for serum enzymes after activation from inactive precursors such as plasmin from plasminogen At least 2 types of serum antiproteases serve to inhibit plasmin (Norman 1957) One serum inhibitor combines rapidly and dissociably with activated plasmin the other more slowly but permanently The net affect is to permit a definite though temporary proteolytic action on suitable substrate Interestingly dilution of fresh guinea pig rat or human serum in glass gives rise in the course of an hour to a nonhistamine permeability factor injection causing a marked

Despite the special and unexpected features that are coming to light through the newer attacks on the problem it still appears likely that the cellular mechanisms of delayed type allergies is immunologic in nature and is connected with the antibody forming apparatus Such allergic responses are characterized by the known immunologic attributes of *specificity* and are capable of *specific desensitization* It is not unlikely that we have to deal with cell bound antibodies of a special sort, perhaps a primitive type of antibody As examples, we may mention dermatitis owing to poison ivy or other contactants and reactions appearing on intradermal testing of patients for current or previous infection when microbial products such as tuberculin or histoplasmin are injected (Table 1)

Owing to these apparent differences in mechanism the 'immediate and the delayed types of allergic reactions will be examined separately Grossly they are not always sharply distinguishable The roles of antibodies and white cells remain rather obscure and allergic excitants can stimulate the immunologic apparatus in both its types of response In clinical tuberculosis for example several circulating antibodies against constituents of the tubercle bacillus appear as *well as delayed type reactivity to tuberculo* proteins

As Table 1 shows some allergic excitants are frank antigens (serum proteins pollen extracts) whereas others are obviously not antigens in their own right Some non antigens (as simple chemical allergens) can be shown to combine with proteins or other substances of the host s tissues as carrier and serve as an artificial antigen at times some alteration in the original structure of the *allergen owing to intermediate metabolism* is probably involved prior to combination It may be helpful to think of antigenic complexes arising from the interaction in vivo between chemical and body constituents as *derivative antigens*

In much the same sense the currently emphasized concept of autosensitization in which an individual is pictured as becoming sensitized to certain of his own tissues probably would require a degradation of native *structure to* the point of incipient antigenicity

The delicate and dynamic balance that exists *in the tissues (homeostasis)* can be upset in ways other than by allergic mechanisms Indeed Roessle has suggested *normergy* to describe the norm of inflammatory responses of normal tissues to a given stimulus *hyperergy* for supranormal reactivity *anergy* for lack of normergic reactivity (owing either to the existence of an immune state or to abnormal cellular physiology) and *hypoergy* has appeared as well commonly to mean a lessened reactivity subsequent to known sensitivity It will be evident that nearly all of the manifestations of allergy are instances of increased levels of reactivity (hyperergy)

Principal emphasis will be given to mechanisms known to be operative in allergic phenomena particularly in animals the *principles then being applied to hypersensitivities* as seen in man *

REACTIONS OF IMMEDIATE TYPE

The allergic responses of animals afford the bulk of our knowledge concerning antigen antibody reactions in relation to tissues Historically, the injection of antigen into actively sensitized animals by the intraperitoneal or the intravenous routes led to *the discovery of systemic shock* (anaphylaxis) whereas injection just below or into the skin led to the finding of *local tissue damage* (the Arthus reaction) Both lines of study have made uniquely additive contributions

The role played by antibodies is proved Immune globulins or antibodies identified as modified gamma β_A and β_{2M} globulins (see Chap 9) are complex in structure and combine with antigen in varying *proportions Some classes of antibodies* over 1 000 000 in molecular weight sediment in a centrifugal field with a speed of 19 Svedberg (19S) units others range around 160 *000 in weight (7S) while some human anti*bodies (reagins) show an intermediate sedimentation pattern somewhat heavier than 7S antibodies Moreover antibodies

* For books presenting special aspects see Lawrence (*1958*) Shaffer LoGrippo and Chase (*1958*) and volumes in the series entitled ***Progress in Allergy*** (Karger New York) and ***Advances in Immunology*** (Academic Press New York)

guinea pig within 10 to 21 days by means of a single injection of soluble foreign protein in the order of 0 1 mcg to 1 mg of crystalline ovalbumin or 0 0001 ml to 0 01 ml of horse serum, preferably injected into the skin With some materials that are less antigenic than native proteins several preparatory injections may be required Because the guinea pig does not form antibody readily and antigens can remain in excess for some time the use of large amounts of native protein antigens can delay appearance of the anaphylactic state for perhaps 6 weeks or more The symptom-complex known as anaphylaxis is demonstrable within 3 to 6 minutes after a 2nd injection of the antigen (0 1 to 10 mg) is given by the intravenous route Alternatively larger amounts may be given by the intraperitoneal route in which case shock often not so acutely manifested starts when enough of the antigen has been absorbed

Within the following minute restlessness is evident the hair especially at the nape bristles often feces and urine are voided the animal scratches at the muzzle with a wiping motion of the forepaws coughs arches its back and raises its head with obvious dyspnea it gives a series of jerks sways goes into violent tonic and clonic convulsions and falls over with cyanosis evident particularly around the muzzle and the ears Death ensues after a few shallow gasps All this occurs often within a period of 3 to 5 minutes Postmortem search reveals firm inflated lungs an actively beating heart active peristalsis and evidences of viceral congestion The trapped air in the lung is seen readily when the organ is cut under water and compressed lightly Coagulability of the blood is found to be decreased

Death from acute shock in the guinea pig is attributable to contraction of the smooth muscle around the secondary and the tertiary bronchioles a prominent anatomic feature in this species The air passages are occluded through infolding of the bronchial mucosa and with lungs remaining distended death results by suffocation Contraction of smooth muscle also explains bristling of hair peristalsis and defecation and contraction of the bladder with involuntary micturition

Lesser degress of anaphylactic sensitivity than the rapidly fatal form described here are to be encountered minimal symptoms include scratching defecation and the characteristic coughs and dyspnea may be transient or not evident

There is another form of shock ("protracted shock") in which dyspnea and bronchoconstriction are much less prominent or even absent this results rather unpredictably usually from subcutaneous or intraperitoneal injection of antigen into the sensitized animal Instead of shock that is explosive in character there is a profound depression at times comalike and oft n with copious tearing accompanied by a marked drop in body temperature (as much as 8° or 9° C) the shock can last for several hours before death or recovery occurs Indeed the guinea pig that exhibits the protracted form of shock instead of acute death is perhaps more informative one sees more drastic changes in blood pressure which after an initial rise falls steadily during the entire period of shock almost complete loss of coagulability of the blood a leukopenia a marked decrease in numbers of blood platelets and a diminution in the titer of "complement (see Chap 9) in the blood owing to fixation by antigen antibody complexes or participation in the activation of plasmin (*vide infra*) When death ensues one observes instead of a ballooning of the lungs edema and hemorrhages of the lungs and congestion of the viscera especially the liver (cf Winter 1945)

In studies of acute shock induced with antigens coupled to radioactive tracers (Warren and Dixon 1948 Dixon and Warren 1950) the antigen causing shock was found to localize around the bronchi particularly in the collagenous tissue between the smooth muscle layer and the cartilage rings this would indicate that specific antibody exists in high local concentration a rather unexpected finding Furthermore these workers found evidence when death failed to ensue until the first 2 to 3 minutes had elapsed of the relaxation of smooth muscle occlusion of the bronchi being then ensured by massive interstitial edema Bradykinin has been demonstrated in guinea pigs undergoing anaphylactic shock reaching a peak at 2½ minutes (Brocklehurst and

but transient increase in the permeability of skin capillaries the substance in guinea pig serum appears to be an α globulin perhaps of protease nature attacking ester linkages meeting in guinea pig serum an α_1 globulin inhibitor (cf Wilhelm 1962) Globulin permeability factor is distinguishable from plasmin but its relation to kallikrein remains unsettled

The relative roles played by these chemical mediators vary from one species to another in relation to amounts available relative sensitivities of a species to particular mediators location of sensitive tissues vis à vis sites of antigen antibody interaction types of antibody and so on For example in the guinea pig we have clear evidence of the *fixation of antibody to tissues with positive* responses obtainable months after antibody *is no longer detectable in the serum a neces*sary period for fixation or equilibration of *passively administered antibody and proba*bly an inhibitory effect of excess circulating antibody in this species histamine in particular appears to play a major role in acute shock although bradykinin may contribute Thus a markedly low susceptibility to anaphylactic shock found in an isologous strain of guinea pig (Wright s Family II) was found to be correlated with a hereditary paucity of histamine in the lung (Stone Liacopoulos Briot Halpern and Neveu to be published) The rabbit in contrast shows only slight evidence of the fixation of antibody to tissues—chiefly in the pulmonary arteries and certain other vessels—but requires circulating antibody in excess *in order* to undergo anaphylaxis Histamine and perhaps serotonin to a degree (Lecomte and Fischer 1958) appears to play the major role in anaphylactic shock *of the rabbit* platelets and basophiles being implicated On the contrary *in sensitized rats in which there* is great insensitivity to histamine (Table 3) *serotonin released from mast cells may* play a relatively important role perhaps *bradykinin also a substance like* SRS-A appears when antigen is injected intraperitoneally Anaphylatoxin shown to be important in dermal reactivity is also produced when *antigen* antibody complexes unite with serum complement The role of the slow reacting substances can be greater in species in which acute shock is not produced quickly

The precipitating feature is union of antigen and serum complement with antibody close to or on the surface of cells It has been suggested that the function of antigen consists in collecting and aggregating gamma globulin molecules such aggregates are pictured as the material causing cellular damage (Ishizaka *et al* 1961) Cells of the vascular endothelium often are affected, becoming swollen apparently phagocytic and permeable allowing rapid development of edema Free and tissue fixed mast cells (Benditt and Rowley 1956 Weiser 1957) and other basophiles (Rorsman Shelley see p 266) are degranulated by antigen antibody *reaction releasing histamine heparin and* varying with the species serotonin Platelets *and white blood cells in the circulation* undergo alterations also becoming attached to the walls *of small vessels and producing* a temporary leukopenia

Heparin preparations have been found to be inhibitory to the release of histamine and serotonin from platelets by thrombin (Humphrey and Jaques 1955) but it appears questionable whether the heparin of basophiles exerts any significant role in the phenomena of hypersensitivity

It is necessary to state that some or all of the end events can be attained in ways that do not require antigen antibody reactions Such phenomena have been termed *anaphylactoid reactions* appearing for example after intravenous injection of bacteriologic peptone or of trypsin or of normal serum either in the pre-clot stage or after incubation *of* serum *with kaolin* starch agar and the like Today as Table 2 shows *we accept the activation* of tissue cathepsins or serum enzymes and their *action on cells and substrates* as the probable pathways Dextran and egg white are known *to release serotonin*

Not all events following combination of antigen with antibody are worked out completely but studies made with the guinea pig are so important that this species will be described in detail

Anaphylaxis in the Guinea Pig The anaphylactic state is readily established in the

guinea pig within 10 to 21 days by means of a single injection of soluble foreign protein in the order of 0 1 mcg to 1 mg of crystalline ovalbumin or 0 0001 ml to 0 01 ml of horse serum preferably injected into the skin With some materials that are less antigenic than native proteins several preparatory injections may be required Because the guinea pig does not form antibody readily and antigens can remain in excess for some time the use of large amounts of native protein antigens can delay appearance of the anaphylactic state for perhaps 6 weeks or more The symptom complex known as anaphylaxis is demonstrable within 3 to 6 minutes after a 2nd injection of the antigen (0 1 to 10 mg) is given by the intravenous route Alternatively larger amounts may be given by the intraperitoneal route in which case shock often not so acutely manifested starts when enough of the antigen has been absorbed

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Lahiri 1962) Possibly bradykinin as well as histamine may contribute to such massive edema Collier *et al* (1960) for example found it usually possible to suppress anaphylaxis by the combined action of an antihistamine (pyrilamine maleate 2 mg/kg) and of aspirin (8 mg/kg) the latter known to mitigate the action of bradykinin on guinea pig bronchioles (aspirin does not antagonize other effects of bradykinin, however)

PASSIVE TRANSFER As mentioned above serum from a guinea pig undergoing active anaphylactic sensitization can passively sensitize normal guinea pigs the recipient animals exhibiting typical anaphylaxis when an injection of antigen is made within the next few days but not before some hours have elapsed

Not only antibody of the guinea pig but in certain instances antibody prepared in other species can sensitize passively e g rabbit The guinea pig is essentially insusceptible to antibody produced by horse cattle chicken or rat Certain human sera can establish passive anaphylactic sensitization—human antitoxic (diphtheric) sera (Neill Sugg and Richardson 1932 Kuhns and Pappenheimer 1952) specimens of serum sickness sera (Longcope and Rackemann 1918 Tuft and Ramsdell 1929) and certain specimens from asthmatic cases (Ramsdell 1930) but human sera containing solely reagins do not do so

Quite recently it has been shown that guinea pig antibodies to soluble protein are separable into 2 types both of molecular weight 160 000 (7S antibodies) but differing in electrophoretic mobility (White Jenkins and Wilkinson 1963 Benacerraf *et al* 1963) Only one of these termed $7S\gamma_1$ appears to be able to fix to tissues and mast cells and give rise to passive anaphylaxis (Ovary Benacerraf and Bloch 1963 cf White *et al* 1963) The other antibody termed $7S\gamma$ seems to arise particularly when certain adjuvants are used Both antibodies are heat stable In the following it must be kept in mind that the quantitative studies cited have been based on total antibody and doubtless adjustments may be in order Likewise it is possible that the similar existence of both slow migrating γ and faster γ_{1A} antibody in mouse horse and so on will yield information on tissue fixation of antibody in these species

The amount of guinea pig antibody that is required to sensitize for fatal anaphylactic shock is extremely small—5 to 30 mcg of antibody nitrogen or less than 0 2 mg of antibody globulin (Kabat and Boldt 1944) The minimal amount of rabbit antibody is of the same order of magnitude—about 30 mcg antibody nitrogen (Kabat and Landow 1942) and 20 mcg of antibody nitrogen has been found to be entirely adequate to ensure maximal sensitization of lung the subsequently isolated lung preparation releasing all available histamine (Liacopoulos *et al* 1963) Rabbit antibody is equally effective whether used as the common precipitating variety or as nonprecipitating or univalent' antibody (Kabat and Benacerraf 1949)

It will be apparent that only a fraction of the administered antibody can become associated with for example a uterine horn removed and perfused prior to use for organ anaphylaxis (*vide infra*) Grabar (1953) has estimated the amount as 0 01 mcg antibody nitrogen

In the guinea pig the anaphylactic state can be inherited from the mother apparently an instance of passive transfer of antibodies as the sensitivity is gradually lost during the first 6 to 10 weeks of life

It has long been known that a latent period of some hours (4 to 18) should be allowed prior to challenge of the passively sensitized guinea pig and from the start the latent period was interpreted as being the time required for fixation of antibody to tissue cells If sufficiently large amounts of immune serum are employed or if one injects soluble preformed antigen antibody complexes prepared in antigen excess (Germuth and McKinnon 1957) no latent period need be observed Benacerraf and Kabat (1949) using quantitative methods and constant antigen found that the amount of antibody needed to result in immediate shock was at least 40 times that required when a 5 hour latent period was allowed and that the sensitizing amount of antibody becomes progressively less as the latent period is extended the minimal shocking dose of antigen decreasing at the same time

Therefore it is understandable that for

passive reversed anaphylaxis in which antigen is injected prior to antibody very large amounts of antibody are required

Passive sensitization can be accomplished by bathing isolated normal tissues in antisera (Dale 1913 Hartley 1939 Kulka 1943 Mongar and Schild 1957a b c Feigen *et al* 1962) including mast cells in pieces of mesentery (Mota 1963) Again the variety of guinea pig antibody that binds to skin and permits PCA tests ($7S\gamma_1$) is the one that sensitizes chopped lung for maximal histamine release $7S\gamma$ proving to be ineffective (Baker Bloch and Austen 1964) The subject has been investigated in detail recently (cf Halpern 1961 Binaghi *et al* 1962) As is seen for sensitization in vivo high concentrations (2 mcg Ab N/ml) of antibody will sensitize in less than an hour but as the exposure time is lengthened increasingly smaller concentrations suffice to attain the same degree of sensitization (0 1 mcg Ab N/ml in 4 hours) In the artificial situation presented here it can be shown that the speed of sensitization is accelerated to a few minutes if the electrolyte concentration is reduced markedly (5×10^{-4}M) or if isotonic nonpolar glucose is used More important it was shown that addition of normal gamma globulin to antibody globulin in the bath (a ratio of 10 1 and higher) reduced the speed of passive sensitization progressively this effect being limited to gamma globulins of those species whose antibody can establish passive anaphylaxis in the guinea pig Likewise the presence of a great excess of nonantibody gamma globulin interferes with passive sensitization with antibody in vivo over a certain time range

Passive Cutaneous Anaphylaxis Instead of inducing passive transfer of the anaphylactic state by means of immune sera injected intravenously or intraperitoneally one may inject intradermally into young guinea pigs (200 to 320 Gm) suitable dilutions of antibody (guinea pig rabbit human) and secure *local* passive sensitization After a suitable latent period such passively sensitized sites can react when antigen is administered systemically or—far more efficiently—intravenously This method of procedure termed Passive Cutaneous Anaphylaxis or simply PCA represents the phenomena of anaphylaxis transposed to and visualized in the dermis (Ramsdell 1928 Chase 1943 1947 Ovary 1951 Ovary and Bier 1953 see review by Ovary 1958) There is an immediate and sharply delimited reaction reaching maximal erythema and edema within 20 minutes and then fading away The technic has been developed chiefly for delicate measurements of antibody (0 03 mcg Ab N/ml or less)

As commonly practiced capillary damage is visualized by mixing with the antigen just prior to intravenous injection dyestuffs that bind to serum albumin—3 5 mg Evans Blue (Ovary) or Coomassie Blue (Feinberg 1963) The local sites develop color within 1 5 to 5 minutes as serum albumin escapes through the injured capillaries the area varying with the concentration of fixed antibody

Just as in passive sensitization in vitro of uterine horn or ileal segment normal gamma globulin can compete with antibody gamma globulin for sites of local fixation along the endothelial wall Hence the technic lends itself best to semiquantitative studies when the concentration of immune globulin in the antiserum is high enough to allow dilution to say 1 30 Further individual differences in capacity to exhibit PCA make it advisable to prepare one site with a standard antiserum of the same specificity as the one under test (Battisto and Chase 1963)

The time for adequate fixation varies with the antiserum Ovary has found standard rabbit antibody to require 4 to 6 hours and Chase has encountered guinea pig antibody that does not react before 9 to 10 hours

Both for passive systemic and for PCA sensitization with guinea pig immune sera there is required the special $7S\gamma_1$ antibody the $7S\gamma_2$ antibody being said not to participate in anaphylaxis but in hemorrhagic Arthus type reactions (p 255) Certain other points deserve mention

(1) Following an intense reaction there occurs a local exhaustion within the area of skin of some component that may not be replaced for as long as 7 days (Chase 1947) probably related to recovery of mast cells (2) Excess antibody introduced into the skin can establish systemic anaphylactic sensitivity and an onset of systemic shock during the test with antigen will negate development of the local reaction (3) Some reactivity remains as long as 5 to 10 days

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Becker and Wirtz (1959) suggested that im mune hemolysis of erythrocytes might offer a useful analogy to allergic events

The same procedure of exposing chopped sensitized lung to antigen or perfusing intact lung with antigen gives rise not only to hista mine but to another pharmacologically active material *Slow Reacting Substance A* (SRS A) (Table 2) Assay is made on guinea pig ileum in the presence of antihista mine drug and atropine Histamine liberation is essentially complete in 5 minutes SRS A appears somewhat later its concentration in creasing for more than 15 minutes Neither platelets nor plasma is required for its pro duction but the synthesis is halted by in terfering with the enzymatic pathway that liberates the preformed histamine Since guinea pig bronchioles are relatively insus ceptible to SRS A this material probably has *no large part in acute shock in this species*

Another pharmacologically active sub stance bradykinin appears in anaphylaxis (Brocklehurst and Lahiri 1962) The action of antigen on sensitized tissue releases a fac tor that acts directly or indirectly to secure enzymic cleavage of plasma α globulins pro ducing a 9 peptide chain which has been synthesized (Table 2) This potent broncho constrictor (cf Collier 1963) has been found to be present within 2 to 5 min utes in anaphylactic shock although it is short lived owing to the action of peptidases The contribution to systemic anaphylaxis of the guinea pig is not determined A general summary is provided in Tables 3 and 4

Anaphylaxis in the Dog Several injections of native protein are usually required to sensitize dogs adequately to prepare for shock although the anaphylactic state may be induced passively by administration of antiserum and reactions can be secured with *out any latent period* (Sherwood *et al* 1946)

Injection of antigen by the intravenous route is followed by restlessness then vomit ing salivation and diarrhea (at times bloody) and finally profound collapse with loss of muscle tone and slow often labored respiration at the same time the blood pres sure and the body temperature decrease markedly As with the guinea pig the re leased heparin causes a loss of coagulability of the blood and there is a decrease in serum complement a pronounced leukopenia in the peripheral blood reflects the elective retention of polymorphonuclear leukocytes in the lung capillaries where masses of white cells adhere tenaciously (Dean and Webb 1944)

In the most acute instances of anaphylactic death necropsy discloses chiefly an enor mously distended and congested liver (in deed it may contain as much as 60% of the blood) for it is the liver that releases the bulk of the histamine (Dragstedt *et al* cf Code 1944) owing to the numerous mast cells in the parenchyma of dog s liver

During shock reactions have been shown in various organs in situ (Manwaring *et al* 1925) contraction of the uterus the in testinal tract (chiefly the colon and the rectum) and the urinary bladder

The *isolated liver perfused with antigen* in Locke s solution will not release histamine and heparin (Scroggie and Jaques 1949) but does so readily when perfused with anti gen in whole blood (Rocha e Silva)

Perhaps affording a most important clue to anaphylaxis in the dog is the observation that 2 classes of canine antibodies can play separate roles passive transfer of serum from dogs spontaneously sensitive to ragweed (see p 263) imposes anaphylactic sensitivity to ragweed extract but demonstration of ana phylaxis is blocked on injecting antiragweed serum prepared in normal dogs (Tennen baum Patterson and Pruzansky 1963) Here the anaphylactic antibody is heat labile and the second one is heat stable

Anaphylaxis in the Rabbit Systemic shock is not experienced regularly Consequently less is known about the conditions for pro ducing systemic shock in the rabbit than in either the guinea pig or the dog The concen tration of circulating antibody must be high (Jackson 1935) Passive sensitization is possible with adequately large volumes of serum (Arthus 1919) and may be demon strated without a preliminary incubation period

Upon intravenous injection of antigen rabbits that show anaphylactic shock show arrhythmic respiration then panting with temporary hyperemia in the ear followed by arteriolar contraction and blanching the rab

in skin prepared with certain guinea pig anti sera, although the sensitivity decreases with time (4) The reactions are usually to be induced far more sharply in the skin of a recipient guinea pig than in the skin of the antibody donor probably because antibody present in the latter's circulation blunts the anaphylactic type reactivity by overlaying an Arthus component

Human skin it may be noted serves for PCA type reactions for a few hours vis à vis certain rabbit immunoglobulins (see p 263) normal gamma globulins compete in fixation to skin so that highly purified rabbit antibody has been required

Organ Anaphylaxis The features of anaphylaxis encountered in the intact sensitized guinea pig are met with also in the individual organs Excised uterine tissue for example contracts on contact with specific antigen even after the organ has been perfused (Dale 1913) and so likewise strips of ileum (Schultz 1910) In the lung bronchospasm and occluding constriction may be demonstrated blood vessels constrict strips of gallbladder contract the isolated heart exhibits an increased rate of beat arrhythmia and constriction of the coronary arteries All these responses are transient the tissue soon returning to the normal state while still bathed with antigen

For detecting the existence of anaphylactic sensitivity as when antibody is not demonstrable in serum the Schultz Dale test can be carried out suspending short lengths of ileum or uterine horns from young females in a 37° C oxygenated bath with Ca^{++} but without carbonate buffer and adding antigen (Fig 1) By this means one can demonstrate readily the complete loss of anaphylactic (but not physiologic) reactivity of the tissue following one maximal response to specific antigen and through this demonstration rule out nonspecific causes of shock

A most important advance was initiated by Mongar and Schild (1957 see review of 1962) who tested in vitro the output of histamine when antigen was added to sliced but not macerated lung fragments of sensitized guinea pigs Below 15° C tissue became desensitized *without* histamine liberation Release of histamine at 37° C was found to involve a heat labile (45°) enzyme precursor (tissue complement) requiring Ca^{++} for activation The short lived enzyme system released bound histamine, probably by degranulating mast cells in the lung Sulfhydryl inhibitors blocked the release Subsequent detailed studies by Austen and Brocklehurst (1961a b c) with various enzyme inhibitors indicated that the enzyme involved resembled chymotrypsin in its specificity becoming active only after the reaction of the sensitized tissue and antigen excluded were cathepsin C carboxypeptidase trypsin, and so on Chymotrypsin itself added to lung tissue had no effect Other evidence suggested that there may be a stage in anaphylactic reaction involving the third component of complement C_3 (Chap 9)

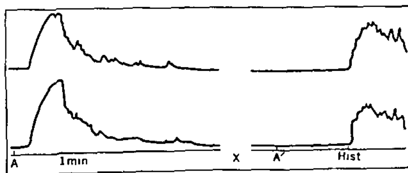


Fig 1 Schultz Dale test Two strips of uterus are tested simultaneously in the same bath *A* addition of antigen to the bath contraction occurring after a short latent period X complete replacement of bath *A'* second addition of antigen to demonstrate desensitization Hist histamine 1 20 000 000 added

(2) Kallidin Lys Bradykinin†		by action of Kallikreins (esterases?) on plasma α 2 globulin			Werle 1960 Pierce and Webster 1961 Greenbaum 1963
Acetylcholine $\left[\begin{matrix} \text{O} & \text{H H} \\ CH_3\,C\,O\,C\,C\,N(CH_3)_3 \\ & \text{H H}\end{matrix}\right]C$	Yes	Bound to protein complex freed by nerve impulses usually Atrium of mammalian heart etc	Parasympathomimetic agent Control of bronchial tone and glandular activity	Irregular	Dale 1914 Went 1936 Farber *et al* 1944 Brocklehurst 1958
Heparin Complex acid mucopolysaccharide as Na+ salt	Yes	Mast cells	Inhibitor of blood clotting	Yes via mast cell degranulation	Gregoire 1946 Lecomte & Hugues 1954
G fraction of plasma (Permeability factors)	Precursors only	α and β globulins	Increase of capillary permeability	?	Wilhelm & Miles 1955 Wilhelm 1956
Leucotaxine Peptide chain *ca* 14 amino acids	No	Cell damage by activated cathepsins	Increase of capillary permeability	Probable	Menkin 1950 1956
Anaphylatoxin"	No	Incubation of Ag/Ab complexes with fresh guinea pig or rat serum involving complement	Mast cell degranulation and histamine release	Probable	Osler *et al* 1959

Reviews *in* Erdos *editor* 1963 Humphrey 1962 Brocklehurst 1962
† Arg argenine Gly glycine Lys lysine Phe phenylalanine Pro proline Ser serine

TABLE 2 PHARMACOLOGICALLY ACTIVE SUBSTANCES IN ANAPHYLAXIS AND ALLERGIC REACTIONS*

COMPOUNDS	PRE FORMED	SOURCE	FUNCTIONAL ROLES	RELEASE BY ANTIGEN/ ANTIBODY	CITATIONS*
Histamine Decarboxylated histidine HN—CH_2CH_2NH (ring: N H)	Yes	Mast cell degranulation Blood platelets (rabbit) Human white blood cells Inducible enzyme histidine decarboxylase (Schayer 1961)	Bronchoconstrictor Capillary relaxant	Yes	Dale 1913 Mongar and Schild 1957 Austen and Brocklehurst 1961
Serotonin 5 hydroxy tryptamine HO—(ring)—$CH_2CH_2NH_2$ (N H)	Yes	Intestinal mucosa Nervous tissue Platelets Mast cells of rodents	Constrictor of gut uterus and smooth muscle especially for rat and mouse	Yes	Humphrey & Jaques 1955 Waalkes *et al* 1957 Levy 1957 Barbaro 1961
SRS A (Slow Reacting Substance A") Acidic substance (complexing with lipids of cell membranes?)	No	Sensitized guinea pig lung perfused with antigen also aorta and great veins	Constriction of guinea pig ileum and rabbit jejunum Bronchoconstrictor for *human*	Yes (from tissues not plasma)	Kellaway and Trethewie 1940 Austen and Brocklehurst 1961 Boreus and Chakravarty 1960 Brocklehurst 1962
Plasma kinins Peptides formed in vivo (1) Bradykinin Nonapeptide has been synthesized† H+ Arg Pro Pro Gly Phe Ser Pro Phe Arg OH−	No	Degradation of pseudoglobulins by enzymes by action of enzyme in pancreas and salivary glands upon activation of proenzyme in plasma slowly by plasmin or trypsin	Bronchoconstrictor Vasodilator of coronary vessels Constrictor of smooth muscle Vasodilator of skin vessels sweat secretion Increases capillary permeability role in edema in allergy	Yes (both tissues and plasma required)	Rocha e Silva *et al* 1949 Antonio and Rocha e Silva 1961 Beraldo 1950 Lewis 1958 Elliott Horton and Lewis 1960 Brocklehurst and Lahiri 1962 Antopol and Chryssanthou 1963

(2) *Kallidin* Lys Bradykinin†		by *action* of Kallikreins (esterases?) on plasma α 2 globulin			Werle 1960 *Pierce and Webster* 1961 Greenbaum 1963
Acetylcholine $\left[CH_3\overset{O}{C}O\overset{H}{\underset{H}{C}}\overset{H}{\underset{H}{C}}N(CH_3)_3\right]C$	Yes	Bound to protein complex freed by nerve impulses usually Atrium of *mammalian heart* etc	Parasympathomimetic agent Control of *bronchial tone* and glandular activity	Irregular	Dale 1914 Went 1936 Farber *et al* 1944 *Brocklehurst* 1958
Heparin Complex acid mucopolysaccharide as Na+ salt	Yes	Mast cells	Inhibitor of *blood clotting*	Yes via mast cell degranulation	*Gregoire* 1946 *Lecomte & Hugues* 1954
G fraction of plasma (Permeability factors")	*Precursors* only	α and β globulins	Increase of capillary permeability	?	Wilhelm & Miles 1955 Wilhelm 1956
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Anaphylatoxin"	No	Incubation of *Ag/Ab* complexes with fresh guinea pig or rat serum involving complement	*Mast cell degranulation* and histamine release	Probable	Osler *et al* 1959

Reviews *in* Erdos *ed or 1963* Humphrey 1962 Brocklehurst 1962
† Arg argenine Gly glycine Lys lysine Phe phenylalanine Pro proline Ser serine

Table 3 Species Differences to Action of Pharmacologic Mediators

	Guinea Pig	Dog	Rabbit	Mouse	Rat	Man
Sensitivity to histamine (lethal*) (mg/kg body weight)	0 3–0 4	3	0 6–3 0	250–300†	170–500 (1 5 mcg i d for local vasodilatation)	
Physiologic acutance to histamine				Adrenalectomy Use of *H pertussis* vaccine	Adrenalectomy	
Role of histamine	Certain	Certain	Principal constrictor	Questionable	Questionable	Certain
Role of serotonin (5 HT)	Not probable		Probable auxiliary role	Certain (Principal constrictor?)	Certain (released in intestine) (Principal constrictor?)	Not probable
SRS A	Produced in lung by Ag/Ab reaction		Produced in tissues by Ag/Ab reaction		Found by Ag/Ab reaction in peritoneal cavity	Produced in lung by Ag/Ab reaction (potent bronchoconstrictor)
Bradykinin	Present in shock (potent bronchoconstrictor)	Present in shock	Present in shock		Present in shock	Possible role
Anaphylatoxin	Can shock				Can shock	
Role of acetylcholine	Improbable	Not probable				Uncertain
G_2 α globulin (permeability factor)	Possible role					Possible role

* Data compiled by the late R L Mayer
† Datum applies to the Swiss albino mice many other strains are more resistant (cf Tokuda *et al* 1963)

TABLE 4 CHARACTERISTICS OF ANAPHYLAXIS IN CERTAIN SPECIES

	GUINEA PIG	DOG	RABBIT	MOUSE	RAT	MAN
Onset of systemic shock	3 to 6 min	10 sec (acute)	15 sec	12 to 60 min		Few seconds
Situs of acute shock	Lung bronchoconstriction	Liver gallbladder G I tract Lungs	Pulmonary arteries mechanical heart failure Liver		G I tract liver congested	Lung
Duration of sensitivity*	Many months	Long vs dog rea in	Brief	Brief	Brief	Many months‡
Fixation of antibody to tissues†	Uterus gut lung vessels gallbladder heart coronary arteries urinary bladder	Brief vs classic antibody	Strips of pulmonary artery blood vessels of lung and ear	Yes slight		Skin lung‡
Incubation period in passive transfer	Several hours	None	None	None		Several hours‡ (Prausnitz Kuestner)
Antibody in excess	Not necessary	Necessary	Necessary	Necessary		Not necessary‡
PCA	Yes (180—320 gm body weight)		Ear only	Yes		Prausnitz Kuestner reaction

* Sensitization established without use of mycobacterial adjuvants
† Reaction to A in saline after perfusion
‡ Reaginic antibody

bit becomes weak and collapses gives a few convulsive movements defecates, and dies suddenly with head retracted and eyes in exophthalmos Bronchospasm is absent but the lung is nonetheless the main organ of shock

At necropsy extreme dilatation of the right half of the heart is seen The inferior vena cava the portal vein and the liver are engorged In *1919* Coca referred the cause of death to obstruction of the pulmonary arterioles which causes right sided dilatation of the heart death being attributable to *secondary heart failure While contraction* of the abundant smooth muscle around the pulmonary arterioles was first regarded as the source of obstruction McKinnon *et al* (1957) emphasized the occurrence of obstructing intravascular amorphous micro thrombi in the pulmonary circulation that in actuality are antigen antibody precipitates as shown by the use of fluorescing antigen Clumps of leukocytes also occur even sufficient to obstruct small vessels The stickiness of leukocytes and the clumping together and clinging of these cells to the vascular endothelium may be observed in newly regenerated blood vessels of the rabbit ear when antigen enters the circulation (Abell and Schenk 1938)

In isolated organs perfused with antigen constriction of a few tissues has been shown (Table 4) but systemic anaphylaxis is attributable to antigen antibody reactions that release histamine and serotonin from the blood platelets and/or leukocytes (Katz cf Waalkes *et al* 1957) probably in close relationship to the pulmonary arteries where white cells and platelets accumulate during shock as mentioned

Liberation occurs also when antibody and antigen are added to normal rabbit blood or to buffy coat the basophile becoming degranulated meanwhile (p 266) and symptoms of shock (nonfatal) can be produced in normal rabbits by injecting antigen antibody precipitates (McKinnon *et al* 1957) In the same way adding preformed antigen antibody precipitates to normal rabbit platelets releases histamine in vitro (Barbaro 1961)

As with other animals temporary anaphylactic desensitization seems to be attained by a few subcutaneous injections of small amounts of antigen in the course of 3 or 4 hours this procedure is employed at times in resuming intravenous injections with certain antigens following periods of rest

Anaphylaxis in Rodents The actively sensitized rat and mouse highly resistant to histamine intoxication (Table 3) undergo systemic anaphylactic shock less readily than other species, and onset is slow Mast cells are injured the chief site of increased capillary permeability is the intestine where enterochromaffin cells appear to be a source of serotonin (Benditt *et al* *1963*)

Upon passive sensitization with rabbit or homologous precipitating antibodies mice and rats give evidence of anaphylaxis but no damage occurs to *their mast cells* (Mota 1963) one is again reminded of the 2 classes of antibodies described in the guinea pig by Bloch *et al* (1963) for perhaps these species too develop in active anaphylaxis a variety of antibody that fixes directly to tissues Indeed the conditions for successful passive anaphylaxis—injection of antigen and antibody closely spaced or presented as soluble antigen antibody complexes (Tokuda and Weiser 1961)—would suggest an anaphylatoxic mechanism as operative by this route Serotonin of high concentration in *rodent mast cells has been implicated* (cf Weiser 1957) both bradykinin and anaphylatoxin may be involved

The rat does not become sensitized readily unless special methods are used (Lipton Stone and Freund 1956) but rabbit guinea pig and potent rat antisera prepare rat skin for passive cutaneous anaphylaxis (Ovary 1958) Indeed Brocklehurst *et al* (1955) showed that PCA reactions were nearly normal in rats 90 per cent depleted of skin histamine by use of the compound 48/80 It is interesting to note that guinea pig 7Sγ *antibody is adequate to prepare PCA sites in* the rat even if not in the guinea pig This has been taken to mean that complement fixing antibody and perhaps anaphylatoxin are important for triggering the reactions of rats (Osler *et al* 1957 1959 Bloch *et al* 1963)

Mice can be sensitized by several injections of fluid antigen (cf Cameron 1956) but much more easily by *alum precipitated*

antigen (Solotorovsky and Winsten 1953) or other adjuvants McMaster and Kruse (1951) have employed the ear of the sensitized mouse for direct observation of vessel spasms during anaphylaxis arteriolar and venous spasm following intravenous injection of antigen is dramatic the capillaries and lymphatics remaining unaffected Mice like rats can be prepared for passive cutaneous anaphylaxis but often reversed passive cutaneous anaphylaxis is employed antibody being injected intravenously and antigen given intradermally 48 hours later

Passive sensitization is possible with potent antisera of rabbit and guinea pig and no incubation period is necessary (Burdon 1946) Several procedures enhance the anaphylactic sensitivity of the mouse adrenalectomy shortly before sensitization or pretreatment with *H pertussis* vaccine (Kind and Parfentjev 1951 review by Pittman 1957) The latter procedure renders mice more than 10 fold as sensitive to histamine as they are normally (Table 3)

Anaphylaxis in Other Animals Anaphylaxis occurs in horse calf and monkey (see p 162 ed 3) Birds may be sensitized and in particular several studies have been carried out in the pigeon the anaphylactic contractability of the isolated circular muscle of the pigeon s crop has been suggested as a laboratory tool

Systemic anaphylaxis in the monkey is not easy to secure The time of reaction to the shocking injection varied from 30 minutes to 24 hours edema and hemorrhage of the lungs and the intestinal tract and of the skin were observed when death did not occur early (see Kinsell Kopeloff *et al* 1941)

Desensitization The introduction of very small amounts of antigen beneath the skin given as a series of injections of increasing amounts over the course of a few hours without the production of symptoms serves to desensitize an animal and to render it immune to shock for some days (Besredka) In desensitization (cf Longcope 1923) it is commonly said that the available antibody is neutralized but some alteration in the reactivity of the tissue component may possibly be involved as well Practical use is still made of this procedure when sensitized animals must receive further injection (Kay 1940) it has been applied successfully to man also but it is hazardous (Friedberger and Mita 1912 Mackenzie 1921 Ratner 1943) *Eventually however with cessation of injec*tions the anaphylactic state becomes re established *there is no known technic for effecting a permanent desensitization*

Inhibition and Shock by Haptenes With the advent of artificial conjugated antigens owing to the work of Landsteiner (Chap 9) it became possible to examine by anaphylaxis the serologic specificity lent to proteins by the attachment of certain types of chemical radicals which have come to be called haptenes When guinea pigs were given sensitizing injections with such a grouping combined with one protein fatal shock could result from subsequent testing with the same grouping combined with an entirely different protein If now the haptene itself or a simple derivative of it was injected intravenously prior to the shocking conjugate a state of specific inhibition of shock was to be found akin to inhibition in vitro of a precipitating antigen antibody system that is the small molecule could compete successfully with the large protein complex for haptene-specific antibody and so delay the requisite interaction between antibody and the full antigen In some cases the effect appears to be a simple inhibition with the tissue retaining its full reactivity in others there is evidence for *specific desensitization as well*

Similarly azodyes in particular those containing 2 haptenic groupings to the molecule (made by such devices as a double coupling to resorcinol) and having probably a larger structure owing to aggregation of molecules of the *dis* azodye can cause anaphylaxis directly without use of protein carriers this matter has been pursued by Campbell and McCasland (1944) who showed further the inhibiting effect of univalent haptenes

Recently haptene amino acid conjugates (termed unifunctional haptenes) have been used to inhibit reactions given by multifunctional haptene protein conjugates with the intent to study specificity For example ε penicilloyl aminocaproate will in excess molar ratio block reactions given by penicilloyl polylysine and by penicilloyl serum albumin (see p 269)

Reversed Anaphylaxis A special instance

described as reversed anaphylaxis is presented by those animal species in whose tissues Forssman heterophil antigens are present for instance the guinea pig If an antiserum produced against some other *heterophil antigen is introduced intravenously* (such as an antiserum against sheep erythrocytes developed in the Forssman negative rabbit) the antibody reacts with the guinea pig s tissues and leads to death with pronounced anaphylactic features however the isolated guinea pig uterus is said not to respond to such antisera

The Arthus Reaction

Antigen antibody reactions that resulted in *localized* tissue damage were found by Arthus in 1903 Rabbits injected repeatedly beneath the skin with horse serum came to respond with progressively more intense reactions at the site of each succeeding injection Eventually Arthus observed hyperemia and edema that were shortly followed by hemorrhage and intense swelling progressing to deep necrosis by the following day later sloughing Study of the Arthus reaction which now is conducted by intradermal injections of antigen into well sensitized rabbits has led to additional knowledge of the effects of antigen antibody reactions in tissues

In the Arthus reaction antigen antibody complexes start forming promptly shortly leading to precipitation in the vessel walls Vascular damage and thrombus formation soon occur but the final consequences—*necrosis of the arteriolar vessel wall hemor*rhage fibrinoid degeneration and edema when the antibody concentration is sufficiently high—require several hours to a day or more

The earlier stages have been studied by Gerlach (1923) Stetson (1951) and Gell and Hinde (1954) Stetson noted pronounced but transient leukopenia within 15 minutes the cells and the platelets being sequestered by adhesion to the endothelium of small vessels aggregates of leukocytes and platelets appeared at the site of injection and formed thrombi Along with vasodilatation and injury to the vascular endothelium swelling of connective tissue fibrils and massive edema occurred locally With the continued increase in numbers of leukocytes at the local site over a period of several hours there was detected an abnormal accumulation of lactic acid owing to aerobic glycolysis by the exudate leukocytes

The acute phase appears to be over by 8 hours (Gell and Hinde, 1954 Cochrane *et al* 1959) Many polymorphonuclear leukocytes are then degenerate still others of them ingest and begin to degrade the antigen antibody precipitate At 8 hours a circumferential mononuclear cell reaction is present In the zone of the mononuclear cells, immature plasma cells and eosinophils are seen by the 4th day and thereafter progression to plasma cells proceeds apace Although several aspects of this mononuclear contribution are seen also in tuberculin reactions of the delayed type the final appearance of plasma cells is confined to the Arthus reaction

Polymorphonuclear leukocytes are essential for inflammatory necrotizing reactions removal of the leukocytes by prior treatment with nitrogen mustard inhibits the Arthus reaction but the Arthus reaction starts up on the eventual return of these cells since the antigen antibody precipitates have not been removed (Stetson 1951)

In relatively avascular cornea slowly developing but marked inflammatory changes can be demonstrated in sensitized rabbits by intracorneal injection of protein (Germuth *et al* 1962) in such sub Arthus' reactions antibody diffuses into the cornea from injured vessels at the limbus producing a local formation of antigen antibody precipitate (cf Movat *et al* 1963) Collagen fibers it has been said become swollen and fragmented but recent studies by Movat *et al* (1963) have not confirmed such alterations The reaction occurs even in animals largely depleted of polymorphonuclear leukocytes by nitrogen mustard treatment

The fixed tissue cells are not themselves sensitive but owe their capacity to react to environmental cellular elements and plasma factors for cells of the sensitized rabbit survive normally in tissue culture when antigen is added

In Arthus reactions ideal conditions apparently exist for antigen antibody complexes to degranulate polymorphonuclear leukocytes intracellular enzymes being freed in

high local concentration along the walls of vessels Platelets give up histamine and serotonin (Table 2) and mast cells in the local site become degranulated The activation of enzymes may bring plasma kinins into play

One newly described factor not discussed above may come into play in tissue reactions that are prolonged over several hours Schayer (1961 1963) has pointed out that histidine decarboxylase is an inducible enzyme contained in the endothelial cells its amount increasing markedly under stress within short periods of time Consequently there is available in an animal not only its preformed histamine but an induced synthesis of new histamine The total amount would not be large but its synthesis for some time in injured endothelium should aggravate vessel permeability increase diapedesis of cells and tend to prolong the local reaction

The intensity of the Arthus reaction varies with the concentration of immune globulin in the serum 120 to 160 mcg antibody nitrogen/ml being required for deep necrosis and slough (Culbertson 1935) Passive transfer of sufficient amounts of immune serum intraperitoneally or intravenously determines typical Arthus reactions upon intradermal injection of antigen and the binding with antigen is found to be maximal within 15 minutes (Benacerraf and Kabat 1950)

A more delicate measurement is reversed passive transfer in which antibody is injected into the dermis and antigen is supplied locally or intravenously Because rabbit antibody does not fix in rabbit skin antigen usually is given intravenously prior to the intradermal injection of antibody The amount of immune globulin needed for reversed passive transfer has been determined as 225 mcg antibody nitrogen for maximal and 24 mcg for minimal reactions (Fischel and Kabat see Kabat and Mayer 1961)

A clear differentiation between anaphylactic and Arthus type sensitivities of the guinea pig was made in 1950 (Benacerraf and Kabat) antibody made both in rabbit and in guinea pig being used In recent studies of Benacerraf and his group (1963) in which guinea pig antibodies are separated into $7S\gamma_1$ and $7S\gamma$ classes it turns out that the 2 varieties of antibody play rather different roles anaphylactic sensitivity is determined by the $7S\gamma_1$ whereas Arthus type reactions are induced—differently—by both The $7S\gamma$ antibody which fixes complement gives rise in reversed passive Arthus tests to hemorrhagic necrosis and $7S\gamma_1$ contributes markedly to the edema (Bloch *et al* 1963) It will be useful to cite the previously described distinguishing features applicable to the use of rabbit antibody as well (1) There is no need of a latent or incubation period before demonstrating passive Arthus reactivity whereas there can be such a requirement for passive anaphylactic sensitization with the lapse of some hours both states coexist (2) The amount of antibody required upon intravenous injection for Arthus type reactivity to be established is considerably greater than that for fatal anaphylactic sensitization (3) Nonprecipitating or univalent rabbit antibody leads only to a low grade Arthus reactivity although it is as effective as precipitating antibody in transferring anaphylactic sensitization (4) Obversely Arthus type sensitivity of moderate intensity is established by injecting antibody of the horse (one reacting with pneumococcal carbohydrate) which is not able to induce anaphylactic sensitivity

The amount of antibody required for reversed passive Arthus reactions in the guinea pig has been measured about 200 mcg antibody nitrogen produces maximal reactions and 10 mcg minimal reactions about the same values as those found in the rabbit (Benacerraf and Kabat 1950) Rabbit antiovalbumin and guinea pig anti ovalbumin were found to be quantitatively equivalent but rabbit nonprecipitating or univalent antibody was markedly inferior

Arthus type reactivity occurs also in monkey (Kopeloff and Kopeloff 1939) horse (Gerlach 1922) and man It is not induced readily in the rat and the mouse except in the lip owing to the number of mast cells found there (Freund and Stone 1956)

A final example apparently the creation of a local Arthus reaction by causing polymorphonuclear leukocytes to concentrate in an area of the skin and there to be affected by antigen antibody complexes forming and

circulating in the bloodstream is provided by experiments of Opie (1936) and Klinge (1933) They showed that localized inflammatory responses may be caused in sensitized rabbits by irritating the skin by xylol or heating or chilling antigen being injected into the circulation

Upon injecting soluble antigen antibody complexes Ishizaka and Campbell (1958) had noted increased vascular permeability in the skin and Cochrane and Weigle (1958) had found Arthus like responses With the thought that this result might be referable solely to gamma globulin molecules held closely oriented in the soluble complex Ishizaka *et al* (1959 1961) aggregated *normal* gamma globulin by heating or by cross coupling with bis diazobenzidine such *aggregates if formed of rabbit or human* gamma globulins indeed gave Arthus type hemorrhagic responses in guinea pig skin and fixed complement to the same degree as soluble antigen antibody complexes Accordingly tissue damage can result from interaction of normal gamma globulin molecules serum complement playing a necessary role Ishizaka (1963) recently has reviewed the role of gamma globulin in hypersensitivity reactions Gamma globulin complexes interacting with serum can activate esterase (from complement fraction C_1) fibrinolysin anaphylatoxin and bradykinin

Localized Tissue Damage

Many workers have injected antigen into sensitized animals in order to explore the pathologic consequences In most experiments of this type the amounts of antigen introduced have been large artificially so in terms of duplicating exposures to antigen that might occur in disease but indeed sites *other than the skin* of sensitized rabbits develop an inflammatory response when antigen is injected locally (e g stomach submucosa testicle kidney and other organs ligatured blood vessels *knee joints pericardium and* myocardium) Thus Klinge (1933) injected antigen into knee joints of sensitized rabbits and produced intense inflammation extending to the adjacent soft tissue with necrosis and collagenous degeneration and repeated injections led to severe destructive and deforming arthritis

Some experiments have approached the problem of disease in more promising fashion Unilateral glomerulonephritis has appeared in sensitized rabbits following the injection of bacterial cells directly into the renal artery (Lukens and Longcope 1931), evidently because of a local retention of bacillary antigen in high concentration

In an attempt to study the genesis of human rheumatic fever Murphy and Swift (1949, 1950) induced successive infections with different serologic types of group A streptococci in a proportion of rabbits subjected to a series of intracutaneous infections over a long period of time cardiac lesions were found that resembled closely those of human rheumatic fever including Aschoff bodies (cf Murphy, 1963) Streptococci *were chosen because of the long suspected* association between rheumatic fever and preceding streptococcal infection Genetic factors appeared to determine the proportion of the rabbits thus affected (In some instances however comparable lesions were found to be induced by large amounts of foreign serum)

Most interesting results have been found in newer studies of so-called Masugi nephritis Here onset of experimental glomerulonephritis is induced by administering low titer specific antikidney antibodies made in other species The foreign gamma globulin attaches to the renal tissue for which it possesses specific affinity constituting there a local concentration of foreign antigenic protein (cf Pressman Korngold and Heymann 1953 Hammer and Dixon 1963) Nearly immediate onset of kidney damage follows the injection of first quality immune sera (cf Hasson Bevans and Seegal 1957 Seegal and Bevans 1957) but such sera in diluted form or sera possessing lower concentrations of antibody require a latent period of 4 to 12 days for the production of host antibody against the foreign gamma *globulin Once initiated the acute* glomerulonephritis can prove to be fatal within a few days or it can undergo remission for several months to be followed by chronic nephritis that persists for months or years The reason for persisting nephritis appears to be deposition of antigen host antibody and components of complement on the base



FIG 2 Section through submucosa of stomach of rabbit subjected to allergic insult showing marked necrosis of medium sized arteries An oblique section of an artery has been exposed revealing 2 segments of its lumen Marked localized damage is evident There is slight infiltration of polymorphonuclear leukocytes in and about the necrotic arterioles Hematoxylin and eosin stain × 75 The histologic finding is typical this particular animal was prepared passively by the slow infusion of antibody after injection of antigen (Germuth F G Jr and Pollack A D 1958 Bull Johns Hopkins Hosp 5 245 Photograph from Dr F G Germuth Jr)

ment membranes of the glomeruli (Fig 4)

Another technic has been to inject into the bloodstream of normal animals very large doses of antigen (0 25 to 1 Gm /Kg protein or 10 ml /Kg foreign serum) the protein being intended both to sensitize and by its persistence in the tissues to provide residual antigen for reaction with newly formed antibodies It will be recognized that this procedure is based on but exaggerates the one that used to lead quite regularly to the development of serum sickness in man when antiserum produced in a foreign species was injected for prophylaxis (antitetanus antidiphtheria serums)

Intensive studies of the pathology produced in normal rabbits following administration of large initial intravenous doses of horse serum were carried out by Rich *et al* (1943 1947) whose studies of fulminating serum disease in man pointed convincingly

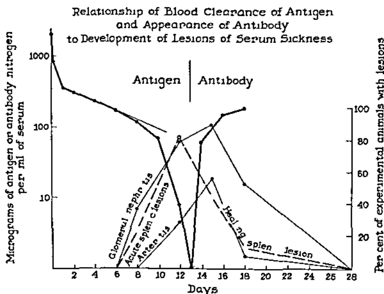


FIG 3 A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type (Germuth F G Jr 1953 J Exp Med *97* 272)

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3) It is evident that the acute lesions—glomerulonephritis arteritis and reversible granulomatous lesions in the spleen and the lymph nodes—occurred during very early antibody synthesis when antibody could be determined only by change in the rate of clearance of antigen from the bloodstream and by consumption of serum complement (Dixon *et al* 1961) (The minimal amount of antibody required to produce this degree of immune clearance was estimated as 2 5 to 4 0 mg of antibody nitrogen in the entire circulation) Next it will be noted that upon disappearance of free antigen and the finding of circulating antibody the various lesions resolved shortly It is evident that the lesions occurred while antigen antibody complexes formed in antigen excess were present and indeed these complexes can be found at the site of damage by fluorescent antibody technics The transient nature of even acute lesions after one injection of antigen and their occurrence only in certain rabbits—the better antibody producers—are evidence of how baffling the problem had been prior to this time

Dixon Feldman and Vasquez (1961) extended this work by giving smaller repeated doses of antigen instead of 1 large injection to a large number of rabbits during many months Here the rabbits suffering from lesions were *not* the better antibody producers (for such animals quickly eliminated each dose of antigen) but the poor antibody producers able to synthesize just enough antibody to form antigen antibody complexes in antigen excess and to retain these for a time in the tissues An example is shown in Figure 4 A and B The lumpy dense deposits along the outer aspect of the basement membrane consist of antigen antibody complement complexes Antigen is localized by staining either with fluorescent antibody or with ferritin tagged antibody (Andreas *et al* 1963) One can draw the conclusion that repeated allergic insults with antigen in persons unable to respond well to the antigenic stimulus may constitute a cause of chronic allergic disease in the tissues

Another example of allergic damage caused apparently by a high local concentration of antigen antibody complexes was provided in artificial manner by Kopeloff and Kopeloff (1949) These workers by introducing antigen into the foot of the guinea pig 3 or 4 days before the onset of sensitivity—established principally from a distant repository of the same antigen in a water in-oil emulsion—produced a most striking inflammatory reaction leading to a chronic arthritic deformity of the foot

Immediate Type Allergies of Man

From the studies made on animals the occurrence of the early type of reaction clearly coincides with the presence of antibodies capable of sensitizing normal individuals the apparent exception being a persisting sensitivity in some animals principally the guinea pig for many months after the antibody has become undetectable in the serum this state is probably due to continued synthesis of antibody in very low concentration followed by fixation to tissues as the transient sensitivity of litters born to female guinea pigs suggests

In man there occur Arthus type reactions anaphylactic shock and localized tissue damage such as glomerulonephritis all related to immunoglobulins We must make it clear at the outset that questions regarding the nature of the antibodies concerned and the subjects capable of producing them are not to be resolved simply So called classic antibodies are produced in man—agglutinins to invading microorganisms and transfused incompatible erythrocytes precipitins to foreign proteins pneumococcal polysaccharides dextrans and so on and univalent antibodies as seen especially in the Rh blood typing system Several other types are known chiefly by studies in man—allergic reagins blocking antibodies and hemagglutinins to pollen extracts

As will be mentioned below the capacity to synthesize reaginic antibodies is supposedly inherited only a small proportion (5 to 15%) of individuals (termed atopic from *atopy* strange disease) exhibiting allergic diseases of the acute type Very many individuals synthesize reaginic antibody in response to horse serum and special materials such as Ascaris extract but the synthesis is apparently transient in contrast with that of atopic individuals

Episodes of acute anaphylactic shock in

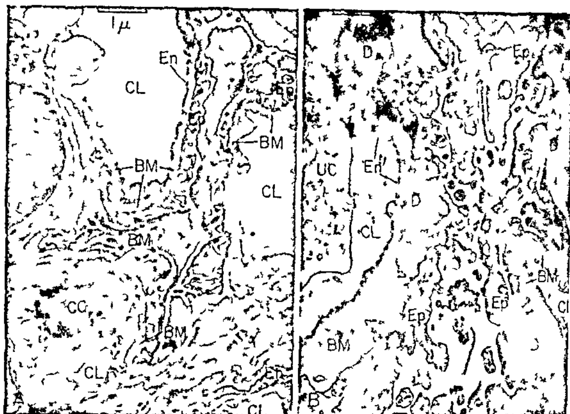


FIG 4 A B Glomerular damage Shown at the same magnification are (A) section through a glomerulus of a normal rabbit and (B) portion of a glomerulus from a rabbit injected with bovine serum albumin daily for 4 months The basement membrane (BM) on the epithelial side has become markedly and irregularly thickened by deposits (D) that are demonstrated through fluorescence microscopy to contain antigen antibody and complement Normal foot processes of the epithelial structure (Ep) are replaced by continuous sheets of cytoplasm (Ep) The inner surface of the basement membrane is flanked by thin fenestrated endothelial cytoplasm (En) becoming more filmlike in the diseased glomerulus BM basement membrane CL, capillary loop Me mesangial areas Ep epithelial foot processes En endothelial cytoplasm D deposits of immune precipitates CC circulating cell within one loop UC cytoplasm of an unidentified cell occupying a large part of one loop (Drs Joseph D Feldman and F J Dixon)

to sensitization as the cause of periarteritis nodosa

Rabbits given horse serum (10 ml /Kg) were sacrificed between 1 and 7 days after the last injection and various types of pathology were found—acute necrotizing arteritis glomerulonephritis of several degrees of severity granulomatous lesions in the spleen and the lymph nodes endocarditis and subendothelial cellular infiltrations (Rich and Gregory 1943) The principle being once established subsequent workers have injected normal rabbits with preformed antigen antibody complexes or have injected antigen intravenously and then infused antibody slowly A typical instance of pathology produced in this way is shown in Figure 2

Most interesting principles emerged when Germuth (1953 Germuth *et al* 1957) and later Dixon *et al* (1961) studied the time at which lesions appeared in relation to antibody synthesis After a single injection of bovine albumin lesions appeared only in those animals that were best able to respond to antigen by early antibody production (Fig

3) It is evident that the acute lesions—glomerulonephritis arteritis and reversible granulomatous lesions in the spleen and the lymph nodes—occurred during very early antibody synthesis when antibody could be determined only by change in the rate of clearance of antigen from the bloodstream and by consumption of serum complement (Dixon *et al* 1961) (The minimal amount of antibody required to produce this degree of immune clearance was estimated as 2 5 to 4 0 mg of antibody nitrogen in the entire circulation) Next it will be noted that upon disappearance of free antigen and the finding of circulating antibody the various lesions resolved shortly It is evident that the lesions occurred while antigen antibody complexes formed in antigen-excess were present and indeed these complexes can be found at the site of damage by fluorescent antibody technics The transient nature of even acute lesions after one injection of antigen and their occurrence only in certain rabbits—the better antibody producers—are evidence of how baffling the problem had been prior to this time

Dixon Feldman and Vasquez (1961) extended this work by giving smaller repeated doses of antigen instead of 1 large injection to a large number of rabbits during many months Here the rabbits suffering from lesions were *not* the better antibody producers (for such animals quickly eliminated each dose of antigen) but the poor antibody producers able to synthesize just enough antibody to form antigen antibody complexes in antigen excess and to retain these for a time in the tissues An example is shown in Figure 4 A and B The lumpy dense deposits along the outer aspect of the basement membrane consist of antigen antibody-complement complexes Antigen is localized by staining either with fluorescent antibody or with ferritin tagged antibody (Andreas *et al* 1963) One can draw the conclusion that repeated allergic insults with antigen in persons unable to respond well to the antigenic stimulus may constitute a cause of chronic allergic disease in the tissues

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man appear to be referable to the human reagin which binds to tissues rather than to other antibodies but it is possible that in stances of delayed shock can involve other types of antibodies

For example Werne and Garrow (1946) reported delayed shock regarded as an in stance of anaphylaxis and certainly related to antibody production Following 2 injections of a prophylactic preparation of alum precipitated diphtheria toxoid and pertussis antigen identical twins died in delayed shock 16 and 24 hours, respectively after the 2nd *injection A role played by hereditary factors* seems to be obvious Both children showed histopathologic evidence of acute vascular injury and edema constricted ar teries and tissue eosinophilia (The prepara tion used for the injections it may be added was shown to produce no untoward reac tion in many other children)

In linking immediate type allergies of man to participation of antibodies it is necessary to point out that antibodies have not been surely demonstrated in for example eczema urticaria (hives) and *angioneurotic edema* Likewise some instances of food allergy can occur without evidence of circulating anti body or cutaneous reactivity to the offend ing foodstuff for these Coca introduced the term familial nonreaginic food allergy Modern developments in biochemistry and in immunology should allow resolution of *these areas for a role of antibodies is most* probable

To connote sites of predilection for allergic reactions seen in various individuals allergists have introduced the term shock organ —the bronchial mucous membrane (asthma) the mucous membrane of the eyes and the upper respiratory tract (hay fever allergic coryza or rhinitis) the endothelium of the superficial vessels in the upper cutis (urti caria wheal reacting allergies) the gastro intestinal tract (forms of food allergy) and the like The implication that antibodies are distributed unequally among the various tis sues suggests a need for critical experimenta tion To some extent the concept of a par ticular shock organ will be an artificiality for the observed reaction may reflect the usual route of contact with the allergen vis a vis the intensity of the sensitivity rather than solely the existence of loci with exaggerated sensitivity For example a person who suffers only from hay fever under natural conditions of exposure will sometimes exhibit instead an attack of asthma or a generalized urticaria when his allergen is *injected for prophylactic* purposes

Arthus Reactions in Man Lesions of the Arthus type can occur in man but are seen only under special circumstances

An excellent example occurred in 1909 (Lucas and Gay) when a decision was reached to sustain immunity against diph *theria in institutionalized children by giving* subcutaneous injections of antitoxic horse serum at 3 week intervals Unwittingly, in view of then current knowledge it was found that between the 2nd and the 6th injections there appeared in addition to scattered cases of generalized urticaria the characteristic local changes—edema tenderness erythema and marked persistent induration or eventual necrosis

Reactions of Arthus type were seen in re cent times in some individuals who received a *single deep intramuscular injection* of peni cillin in a beeswax and oil vehicle Appar ently, antibodies arose to some component of the injection mass and reacted with the antigen still partly held in situ

Therapeutic injections of immune horse serum given to severely ill, sensitive patients have at times produced extraordinarily ex *tensive reactions and sometimes have* even showed black dry eschars over large areas (Gatewood and Baldridge 1927 Tumpeer *et al* 1931) Extensive slough of the skin and the subcutaneous tissue has been noted (Kohn McCabe and Brem 1938) Whether or not there is some auxiliary role attribut *able to concomitant bacterial disease is un* known—for example participation of the Sanarelli Shwartzman phenomenon (p 293) Passive transfer of local serum reactivity in man has been reported by Kojis (1942)

Serum Disease * After the discovery and the clinical application of diphtheria anti toxin *the illness known as serum sickness* came to attention Patients treated by thera peutic injections of a single dose of antitoxic

* The monograph by von Pirquet and Schick (1905) is comprehensive see Longcope and Wink enwerder (1941) and Ratner (1943)

horse serum showed within 7 to 12 days—closely similar to the time required for the development of the anaphylactic state in the guinea pig—widespread urticarial or erythematous eruptions with intense itching a generalized swelling of lymph nodes and often edema of the eyelids the face and the ankles In severe cases arthralgia and fever followed the eruption

The disease appears as a consequence of the interaction between specific antibodies newly formed and remaining traces of foreign proteins The disease may be transient (the usual period is slightly over 2 days) or it may last up to 2 weeks With use of crude horse serum there were usually several spontaneous recurrences of the eruption attributable to the fact that antibody formation is established at different times to the various serum proteins Hawn and Janeway (1947) showed for example that serum sickness following injection of isolated beef gamma globulin occurred in 7 days and in 14 days when beef albumin was employed alone Similarly in rabbits the times for beef gamma globulin and beef albumin to induce pathologic changes after single injections followed the pattern seen here for human serum disease owing to the differential rate of catabolism of these proteins gamma globulin produced chiefly kidney and nonarterial heart lesions and albumin caused arterial lesions (Hawn and Janeway 1947 Heptinstall and Germuth 1957)

The incidence of serum sickness today is reduced because of a lessened use of sera of animal origin and because of chemical modifications to which some immune globulins can be submitted (e g partial cleavage with pepsin as in the *Parventjev process cf Rose et al* 1962) Antibody preparations to tetanus toxin were long used as a prophylaxis following wounds but the present trend is to immunize in the early years with tetanus toxoid and also with diphtheria toxoid Nevertheless it was estimated that 136 000 doses of tetanus antitoxin were administered in Canada in 1 year (Toogood 1960) with 7 900 allergic reactions

In serum disease antibodies have been found in patients sera, giving precipitation in vitro with horse serum anaphylactic sensitization in the guinea pig and tanned cell hemagglutination reactions versus horse serum (Longcope and Rackemann 1918 Tuft and Ramsdell 1929 Arbesman *et al* 1962) and like findings appeared in certain allergies to common foods (*Schloss 1912*) These antibodies being precisely those that were to be expected in view of the antigenic stimulus the disease came to be regarded as either an anaphylactic or an Arthus type allergy as would be consonant with the involvements of joints and lymph nodes (cf Table 1) At the same time skin sensitizing antibodies having the characteristics of reagins often are present (de Besche 1923) and perhaps are responsible for the urticaria and the itching Arbesman *et al* (1962) found that reagins arose with the onset of clinical symptoms preceding the rise of antibodies that are measured by hemagglutination and falling while the antibodies demonstrable by tanned cell hemagglutination still persisted (Similarly transient display of wheal and flare reactions but persistence of hemagglutinins was found by Fisher and Connell (1963) upon actively sensitizing a normal nonatopic volunteer with ragweed extract) Clearly both skin sensitizing reagins and classic antibodies occur together In this regard it may be mentioned that the antibodies present in serum sickness can be demonstrated advantageously by reversing the sequence of injections used in the Prausnitz Kuestner technic probably because antibodies other than reaginic skin sensitizing antibody can participate (Voss 1938 Wright and Hopkins 1941 Karelitz and Glorig 1943)

In predicting which patients would develop serum sickness Arbesman *et al* (1962) *stated that the greatest correlation* was observed with patients having pre injection titers against horse serum-coated tanned erythrocytes greater than 1 200 by their method

Reinjection at a time when the sensitivity is sufficiently high may cause local immediate reactions within 15 minutes to an hour and also general immediate reactions occurring within some minutes to 12 hours generalized immediate reactions often are severe and occasionally may prove to be fatal Further mention will be made below

If the antibody producing mechanism has

been established by some previous but not too recent injection a new administration of the same foreign protein will bring on the original train of events after a shorter interval 3 to 5 days (accelerated reaction) paralleling the recall phenomenon observed when animals are restimulated with antigen after a rest period

In recent years clinical manifestations closely resembling serum disease have been seen as a consequence of drug therapy for instance with the sulfonamides, the arsphenamines penicillin and others (Longcope, 1943 cf Carr 1954) A formation of derivative antigens in vivo between tissue component and chemical probably explains the sensitizing effect and could account for the frequent failure to find antibody or to reveal skin sensitivity by use of the chemicals themselves The pattern of serum disease is manifest in the occurrence of fever skin eruptions (erythema urticaria or purpura) and lymphadenopathy and as an aftermath of sensitization both accelerated and immediate types of response are to be encountered upon readministration of the drug

Anaphylaxis in Man Reactions that were judged to be anaphylactic in character were noted in the older experiences with foreign serum as during the course of spaced intrathecal injections of antimeningococcal horse serum employed therapeutically for meningitis Most of the fatal and grave incidents encountered in administering horse serum occurred in people having a pre-existing clinical *sensitivity to horse dander that is horse* asthmatics (Ratner and Gruehl 1929) and again it is the reaginic antibody and atopic individuals that are implicated Most prone to systemic accidents were those who gave immediate flare and wheal reactions in skin testing (This statement holds also for persons sensitive to insect stings e g bees wasps)

An ostensible instance of reversed anaphylaxis not dependent on reagins was seen in uterine contractions following the administration of specific horse antipneumococcal sera to women ill with lobar pneumonia and consequently possessing pneumococcal polysaccharide

In recent years fatal reactions to penicillin (p 269) have been noted as well as to bee venom foreign protein and pollen extracts all of these being related to the allergic state and probably to a reaginic mechanism (cf James and Austen 1964)

Longcope and Winkenwerder (1941) reviewed the severe reactions occurring after administration of horse serum—collapse fall of blood pressure tachycardia dyspnea of the asthmatic type suffusion of the face urticaria with giant wheals and sometimes marked edema of the entire body in fatal cases death would occur within a few minutes or as late as 24 hours Postmortem examination was apt to reveal distended lungs splanchnic congestion and at times engorgement of the liver

Quite recently James and Austen (1964) examined 6 instances of fatal anaphylaxis in which death ensued within 16 minutes to 2 hours Hyperinflation of the lungs was encountered in the 5 cases presenting respiratory symptoms Both grossly and microscopically there was obstructing edema of the hypopharynx epiglottis and larynx in 4 patients, and microscopically there was acute emphysema in all 5

One lesson remains to us whenever it is deemed advisable to inject a foreign protein the possibility of pre existing sensitivity must be kept in mind Testing for immediate type sensitivity is generally practiced by cautious intracutaneous injection and conjunctival instillation of *dilute material* waiting up to 2 hours for evidence of a reaction prior to therapeutic administration of antisera of *animal origin* As Longcope emphasized (1943) there should be employed in addition a preliminary intravenous test with 0 1 ml or less of the material untoward reactions may occur in the absence of positive cutaneous and conjunctival tests as is comprehensible in view of the several varieties of antibody mentioned above

Allergic Coryza (Hay Fever) The prompt response that occurs in patients who encounter pollen to which they are sensitive and their physiologic reactions when pollen extract is injected in too high a concentration constitute a prime example of anaphylaxis in man (The other chief example is afforded by insect stings which will not be treated here) The parallelism between human and guinea pig anaphylaxis is shown in Table

TABLE 5 COMPARISON OF HUMAN ANTIBODIES ENCOUNTERED IN ALLERGIC CORYZA AND ASTHMA

	REAGIN	CLASSIC 7S ANTIBODY
Heredity implicated	Yes (Atopics)	No
Antigenic stimulus	Natural exposure (spontaneous)	Via injection (traces by natural exposure)
Protein type	β_A globulin also others (?)	γ globulins
Ultracentrifugation $S_{w\,0}$	8 to 11S	7S
Heating at 60 C	Labile	Stable
Reaction in vitro with antigen	Allergen bound to immune precipitates of reagins (??)	Precipitation shown by special methods in patients serum
Passive sensitization of skin	Fixes to skin mucous membranes of nose eye intestine	Leaves skin readily
Prausnitz Kuestner test	Yes	No
Placental passage	No	Yes

4 Because of the danger of anaphylaxis associated with the injection of specific allergens dilute solutions are used and epinephrine is kept ready the latter both relaxes smooth muscle and contracts some of the peripheral vessels

The antibody responsible is the human reagin * (1) It fixes to normal skin upon passive intradermal transfer and gives flare and wheal reactions when the corresponding allergen is injected intradermally into the same site (Prausnitz Kuestner 1921 the P K technic) or is absorbed following injection of larger amounts into remote tissues (Lippard and Schmidt 1937) Injected sites can give some measure of response when tested initially as long as 45 days later (2) The antibody differs from classic antibodies (Table 5) in several respects it is heat labile (56° to 60 C for 1 to several hours) it fails to show visible reaction in vitro with the corresponding allergen it is associated with β_A globulins and perhaps with other serum globulins but not with the classic gamma globulins it is dissociated by mercapto-ethanol (Leddy *et al* 1962) It is absent in children born to allergic mothers and it is not able to sensitize guinea pigs anaphylactically even though other human antibodies can prepare sites for passive cutaneous anaphylaxis in guinea pigs

Its prototype has been found in a few dogs suffering spontaneously from allergic coryza (Wittich 1941 Patterson 1960) in certain cows (Reddin 1948) in rabbits (Sherman *et al* 1939 cf Vaughan and Kabat 1953 Aladjem *et al* 1957) and probably in guinea pigs (Caulfeild Brown and Waters 1937) as judged by heat lability The rabbit and the guinea pig antibodies are assigned to the class of reagins principally because of demonstrated fixation in human skin Intensive studies of dogs spontaneously allergic to ragweed are presently being conducted Transfer of dog reaginic serum imposes anaphylactic sensitivity as well as cutaneous sensitivity and asthmatic attacks occur by inhalation of the allergen (Patterson and Sparks 1962 Tennenbaum *et al* 1963)

It should be noted that isolated specific rabbit immunoglobulins (prepared to dinitrophenylated proteins and to penicilloyl bovine gamma globulin) will serve to sensi

* Reagins or less precisely skin sensitizing antibodies are not to be confused with Wassermann reagin which for long has been a designation for the syphilitic antibody

tize and to produce wheal and flare reactions in human skin the nonreaginic antibody having a half life of only 10 hours (Farah Kern and Eisen 1960 Parker *et al* 1962)

The flare and wheal reaction appearing in sensitive persons by scratch testing through drops of allergenic extract or more exactly by injecting graded doses intradermally, consists of 3 developmental phases duplicating those seen upon injecting histamine into human skin (the triple response described by Sir Thomas Lewis) (1) local capillary vasodilatation producing an initial erythema (2) a local axon reflex causing widespread *arteriolar dilatation and producing a flush* or flare spreading gradually from the center (3) an increase of permeability in the integrity of minute blood vessels allowing very rapid passage of fluid with development of a wheal (a sharply circumscribed and elevated blanched central area sometimes showing pseudopodial extensions) The entire area involved may extend from 3 to 10 or more centimeters in diameter with a wheal of 1 to 3 centimeters The reaction occurs within 5 to 30 minutes and usually begins to fade *within a further 15 to 60 minutes after which* the skin nearly regains its normal appearance

Not every person synthesizes reagin under conditions of natural exposure those who do so *being classed as atopics It is widely be*lieved that the synthesis of reagins is an inherited capacity (Cooke and Vander Veer 1916 Spain and Cooke 1924) indeed only between 5 and 15 per cent of the population suffer from atopic diseases and it seems that hay fever (with asthma) runs in families The inheritable property is a susceptibility for sensitization only since the allergens concerned and the type of clinical manifestations vary among sibs and between children and their parents

The development of reagins or the reaginic type of skin reactivity in a sample of the population has been observed several times under conditions of natural exposure e g, to dead sewage flies (Psychoda) (Ordman 1946) and to castor bean pomace (Figley *et al* 1928 1950) The small proportion of reactors among those exposed is at least consonant with the idea of inherited susceptibility At the other extreme is the formation of the reagin type of antibody by many persons upon adequate artificial immunization with selected materials namely, extracts of Ascaris (Rackemann and Stevens 1927 Davidson Baron and Walzer 1947) and of Trichina (Baron and Brunner 1942) and horse serum (p 261) Apparently in an intermediate position between these 2 groups stands the relatively high proportion—at least 40 per cent—of persons who develop flare and wheal reactions to an impurity contained in diphtheria toxoid in consequence of a single booster dose of partially purified toxoid (Kuhns and Pappenheimer 1952 Relyveld Henocq and Raynaud 1961) The problem is difficult e g among bakers in Denmark *developing clinical sensitivity to flour no evi*dent familial susceptibility could be established (Schwartz 1952)

THE PRAUSNITZ KUESTNER TEST The passive sensitization of normal volunteers with patient s serum remains the only sure identification of the human reagin In sensitizing passively one sees the same relationship as in establishing passive anaphylaxis in the guinea pig a given amount of reaginic serum will produce markedly greater sensitivity if a latent period of 1 day is observed but *it is possible at the cost of working* with submaximal reactions to obtain immediate reactions by injecting allergen and reagin together when the latter is sufficiently concentrated *A more compelling reason to observe* a waiting period is to avoid misinterpretation since immediate whealing can be produced in human skin by the toxic effects of certain sera and by mechanical trauma * As with all transfers of human serum the danger of serum hepatitis must be weighed

Human reagins fix in the tissues of monkeys Walzer having used rhesus monkeys for passive sensitization of the stomach submucosa and so on (see Walzer 1941) Rather recently it has been shown that the *Macaca irus* monkey can give useful passive sensitization of the skin (Layton Lee and DeEds 1961) and some workers are using such animals as substitutes for human volunteers It is necessary to elicit locally prepared sites by *intravenous* challenge with Evans

* Immediate whealing is produced in normal individuals upon injection of aggregated human gamma globulin formed by heat or coupling via bis-diazotized benzidine (Ishizaka *et al* 1961) The activity of such aggregates appears to be related to their interesting property of binding serum complement

Blue and allergen and monkeys can be reused several times at intervals of 2 weeks before they become refractory to human reagin An intravenous injection of as little as 3 ml of human reaginic serum prepares the animals for general cutaneous reactions and for ex hibiting typical asthma by inhalation of the corresponding pollen

There is a high correlation between reagins and clinical disease in wheal reacting aller gies but failure to detect reagins by passive transfer (P K) in the circulation does not mean the absence of disease upon exposure owing perhaps to continued synthesis in trace amounts and tissue fixation of reagins

One unresolved problem is that reagins have been found in individuals who show no corresponding clinical allergy (Grow and Herman 1936) judgment having been made on the basis of history (natural exposures) and not under experimental conditions This finding is consonant with the finding by aller gists of flare and wheal reactions in diag nostic testing that may be deemed of no clin ical significance Rather case histories are sought relative to allergens showing the strongest responses upon testing and treat ment may be confined to the use of several allergens or only those for which the case history indicates clinical disease In a few instances reagins have been detected 1 to 3 years before the onset of a corresponding clinical allergy

Several questions might warrant evalua tion (1) whether in addition to reagin some special concentration of it or alteration in a particular tissue site (shock tissue) is required to establish a clinical disorder (2) whether reagins can exist of low avidity (or affinity) that would give rise to less physio logic disturbance in mucous membranes than would be expected from their whealing re actions in the skin

HISTAMINE RELEASE FROM WHITE BLOOD CELLS In the last few years knowledge has grown concerning the multiple antigens of ragweed and grass pollens * these being sub jected to separation by modern biochemical technics and studied by immunoelectrophore sis versus rabbit antisera Aqueous ragweed extract for example contains more than 13 antigenic components not all of these being related to clinical sensitization As purer products become available it is found that very dilute solutions of certain fractions of *short ragweed as* 5×10^{10} *mg/ml induce* whealing in sensitive skin less than a 1 000th the amount of crude extractives (King and Norman 1962 King Norman and Connell 1964) Some ragweed-sensitive individuals appear to have coexisting sensitivities to more than a single constituent, since P K test sites after exhaustion of the capacity to react with purified material can still respond to whole extract (Arbesman *et al* 1962) The preparation of rather pure allergenic sub stances which are present only in small concentration in whole extract allows much more critical tests to be made than formerly were possible For example the coating of red cells with such products gives much higher titers than when many substances compete for the surface of the erythrocyte

Lichtenstein and Osler (1963) using the highly purified Antigen E of King *et al* (1962 1964) have been able to show his tamine release from the washed blood cells of ragweed sensitive patients at an allergen concentration of only 10^{-3} mcg/ml or less thereby confirming with 100- to 1 000-fold less material the pioneer findings of Van Arsdel and Middleton *et al* (1958) Middle ton *et al* (1960) VanArsdel and Middleton (1961) The antibody that is attached to the circulating white cells of allergic patients ap pears to be reagin both because it has been seen to decrease upon hyposensitization (VanArsdel and Middleton 1961) and be cause reaginic sera can sensitive passively normal human erythrocytes providing that serum is held at —70° C or is fresh (Van Arsdel and Sells 1963) Augustin (1962) concluded that reagins absorb both to nor mal leukocytes and to sheep erythrocytes The ratio of allergen to white blood cells is critical less histamine appearing when aller gen is in excess Divalent cations are re quired All human leukocytes were found to contain a soluble product which enhances *histamine release in this system*

BASOPHILE DEGRANULATION TEST In 1957 Rorsman noted that the number of

See Augustin and Hayward 1961 (orchard grass timothy pollens) cf Richter and Sehon (1960) Goldfarb and Callaghan (1961) King and Norman (1962) and Robbins *et al* (1963) on short ragweed pollen

basophilic leukocytes was decreased in the blood of patients with urticaria, he suggested that antigen antibody reactions or histamine liberating substances might degranulate these cells Subsequently Rorsman studied basophiles during allergic reactions of rabbits (1961) and guinea pigs (1962) In particular, he found that basophiles decrease if the anaphylactic shock of the guinea pig is mild or only moderately severe Shelley approached the problem by seeking to demonstrate basophile degranulation in vitro Shelley and Caro (1962) indeed found that addition of antigen (1% ovalbumin) to blood of sensitized rabbits degranulated a significant proportion of the basophiles in vitro and that these cells disappeared from the blood upon systemic shock

Turning to human allergic sera Shelley devised several technics to allow diagnostic use of such a test (1962 1963) The *indirect method* consists of using rabbit basophiles from selected New Zealand rabbits having 8 to 10 per cent of these cells passively coating the cells with sera of penicillin sensitive patients and observing the basophiles delineated by the vital dye neutral red for degranulation during exposure to allergen Positive results were reported in a narrow time range (1963a b) Several other types of hypersensitivity were tested with reports of positive results Although the method holds promise and many laboratories are engaged in study it is not presently perfected technically and indeed passive sensitization of rabbit cells with human reagins offers difficulty Some claims such as that prompt degranulation follows simple admixture of aspirin with basophiles and sera of aspirin sensitive patients deserve critical study

NONREAGINIC ANTIBODIES DESENSITIZATION Under natural conditions of exposure to pollens the allergic individual—the so called pretreatment patient —long had been thought to produce reaginic antibodies solely, these antibodies being multiple and corresponding to one or several of the allergenic substances within a given pollen

Yet with introduction of the passive hemagglutination technic in which the allergens are attached to the erythrocyte surface by diazotization through a benzidine bridge (via bis diazobenzidine or BDB) or are coated onto tanned red blood cells it was found that pretreatment patients possessed antibodies that agglutinated the erythrocytes to varying degrees but failed to precipitate ragweed proteins in vitro (Gordon Rose and Sehon 1958) If the serum globulins were concentrated 10 to 20 fold however direct precipitation was demonstrable (Perelmutter *et al*, 1962) Subsequently it became possible largely to separate the hemagglutinins apparently having properties of divalent precipitating antibodies from the reagins (Sehon 1962)

When allergic individuals have been subjected to desensitizing injections with their allergens in gradually ascending tolerated concentrations their posttreatment sera have been found to acquire a heat stable antibody designated as blocking antibody that combines readily and specifically with the allergenic material (Cooke *et al* 1935 Loveless 1940) The thermostable antibody neutralizes the allergen becomes a mixture of the two will fail to produce a positive skin test on a sensitive individual (Loveless 1940)—hence the name blocking or neutralizing antibody (Normal nonatopic individuals under the same treatment give rise somewhat less readily than allergic individuals to the same type of antibody reagin does not appear in the circulation but under intense antigenic stimulation Fisher and Connell (1963) observed a wheal and flare reactivity of transient nature)

The higher titered specimens of blocking antibody were found to bind with allergen in complement fixation tests (Chap 9) then by diffusion in gel media these antibodies were seen to precipitate the allergen(s) Today the blocking antibodies are equated with the hemagglutinins mentioned above the apparent differences between nonprecipitating blocking antibody precipitins and hemagglutinating antibody being attributed to the concentrations in which they occur in different sera

In light of the recognition of blocking antibody as dilute precipitating antibody and the large number of antigens in ragweed that incite separate antibodies in rabbits it seems obvious that man under desensitization treatment will produce a variety of precipitins toward different constituents

Study of various aspects of the problems of desensitization doubtless will require that separated allergens be available

The presence of blocking antibodies in the serum of allergen treated patients can be determined after heat inactivation of the reagins by in vivo testing Conversely since blocking antibodies readily leave the skin after injection whereas reagin does not it is possible to measure reagin separately In order to test blocking antibody in vivo mixtures made with allergen are injected directly into sensitive or passively sensitized skin (The concentration of blocking antibody does not rise sufficiently high in patients possessing it to influence the occurrence of wheal and flare reactions upon cutaneous testing with allergens)

The idea that the process of desensitization is the development of a type of antibody other than the reagin the former protecting the patient by isolating newly absorbed allergen from sensitized tissues is still to be decided The complexity of pollens make the issue hard to resolve A more favorable situation for deciding the issue is offered by cases of insulin sensitivity coexisting reagin and thermostable antibody have been encountered the thermostable antibody appearing indeed to be responsible for periods of insulin fastness (Lowell 1944 1947 Loveless 1946 Berson *et al* 1956)

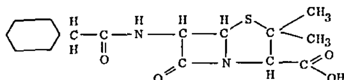
In attempting to desensitize the allergist is limited to the administration of very small amounts of pollen extract lest systemic accidents result The administration may be preseasonal in the case of flowering plants or it may be maintained throughout the year (perennial treatment) The idea that depot treatment—injection of large amounts of allergen in water droplets emulsified within a continuous phase of paraffin oil—would allow slow release of a greater amount of allergenic solutes and be more efficacious has come to the fore (Loveless 1957 Brown 1959) Unfortunately the idea of one shot injection therapy has dominated rather than a careful appraisal of the possible merits of the new type of treatment utilizing several injections Owing to improper emulsification or to instability of stored emulsions free allergen has been found at times in dangerous concentrations Nevertheless the method has yielded results that compare favorably with standard desensitizing procedures and study continues Other problems can arise as by provoking delayed type hypersensitivity to allergens p 287

NATURE OF THE REAGINS The thermolabile reagins in serum as has been seen above are accompanied by other immunoglobulins and they are dilute one estimate of the maximum concentration is 100 mcg / ml in those few sera that can give P K tests in 1 1 000 dilution (Gyenes and Sehon 1960) All early methods of fractionation indicated that reagins become more widely distributed over crude fractions than precipitins Further they have proved to be labile during biochemical fractionation and recovery of activity is low Again the number of specimens of reaginic serum that have been examined by modern methods is not large there is strong suspicion that a certain number of ragweed sensitive patients are sensitized to more than a single allergen of the extract The available evidence associates at least the major reagin of ragweed serum with β_A globulin and appears to exclude participation of β_M macroglobulin (19S) Some allergic individuals however are said to lack β_A globulin

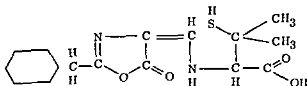
Heremans (1960) and Augustin (1961) suggested the relation of reagins to β_A globulin and indeed Fireman Vannier and Goodman (1963) found reaginic activity to follow β_A globulins as they fractionated serum from treated ragweed sensitive individuals obtaining 10 per cent recovery Selective removal of β_{2A} globulins from entire serum by means of specific sheep antiserum gave a marked decrease in reaginic activity whereas a similar selective removal of gamma globulin left the original titer unaltered Ishizaka *et al* (1964) have found β_A globulins as the sole protein of the parotid duct along with the presence of reagin It may be added that most workers have found reagin to elute in column chromatography just ahead of gamma globulin this finding would be consistent with the determination of Rockey and Kunkel (1962) that the skin sensitizing antibody reacting with a highly purified glucagon sedimented faster than 7S antibody perhaps between 8 and 11S In

this case the reaginic property was inactivated by 0 1 M mercapto ethanol

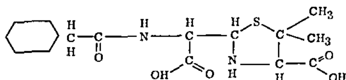
In a new technic (Yagi *et al*, 1963), the proteins of reaginic serum were separated by electrophoresis and then precipitated in situ by antibody to human serum I^{131} labeled allergen being present Labeled allergen became bound to the precipitate its position being detected by autoradiography Insulin became bound to gamma globulin and at times to β_A globulins and a rather purified sample of ragweed allergens became bound to β_{2A} globulins and to gamma globulin and sometimes to β_M macroglobulin (Yagi *et al* 1963b) Obviously, it is not possible to know the participation of hemagglutinating and other nonreaginic antibody in these precipitates The same technic was applied in an



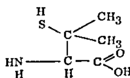
Benzylpenicillin



Benzyl penicillenic acid



Benzyl penicilloic acid



Penicillamine

FIG 5 Some reaction products of penicillin G

other form by Pruzansky *et al* (1962) a large amount of a highly purified I^{131} ragweed allergen binding in vitro to a precipitate formed by mixing reaginic serum directly with antihuman gamma globulin

REAGINS TO PENICILLIN Even though the more common form of hypersensitivity seen with penicillin is a delayed reaction resembling that of serum sickness explosive systemic reactions related to the presence of reagins can present grave danger and result in death Within 2 or 3 months after shock circulating reagins disappear the patient retaining cutaneous reactivity In testing actively sensitized persons or passively sensitized sites potassium penicillin G usually induces wheal and flare reactions (Schwartz and Vaughan 1963 VanArsdel *et al* 1963 Siegel 1962) but the reagins of 1 patient reacted only to stored procaine penicillin G solutions Of the proposed pathways through which (benzyl)penicillin reacts with host tissues successive conversions to penicillenic acid and then to penicilloic acid (Fig 5) have been postulated (Levine 1960 1961 Parker de Weck Kern and Eisen 1962) protein reactions occurring both via ε amino lysyl groups and SH groupings the latter products involving the SH of penicillenic acid and forming mixed disulfides

In order to detect immediate type reactivity to penicillin penicilloyl human gamma globulin conjugates have been employed in intradermal tests (Levine and Ovary 1961 Parker *et al* 1962) The most extensive investigations have been made with penicilloyl polylysine some preparations of which have contained not only penicilloyl groups but also a few penicillenate groups and penamaldate penilloaldehyde groupings newer preparations are virtually free of any but penicilloyl groupings (Parker Shapiro Kern and Eisen, 1962 Parker and Thiels 1963) Reactivity is expressed almost exclusively to the penicilloyl groupings and accordingly inhibition of cutaneous reactivity is usually demonstrable with ε penicilloyl aminocaproate (Recently Parker and Thiels 1963 have suggested that conjugates with polylysine made from the unnatural (D) amino acid may prove to be safer with respect to nonantigenicity of the complex) Yet about one half of reacting individuals respond to penicillenate conjugates as well reflecting the complexity of degradation reactions of penicillin in vivo When it is considered mandatory to make intradermal tests with penicillin G in order to reveal the most sensitive persons great caution must be exercised with use of scratch tests with 1 unit followed by intradermal tests with 1 5 and slightly higher unitage Only a few persons react in this way in contrast with the numbers reacting to tests with polylysine conjugate

In addition to reagins other types of antibodies to penicillin—both 7S and 19S—can be found in serum capable of agglutinating penicillin treated erythrocytes (Ley *et al* 1958 Schwartz and Vaughan 1963 Van Arsdel *et al* 1963) and fixing in guinea pig skin (PCA reactions) Indeed many individuals given penicillin form antibodies that can be detected by hemagglutination but without concomitant symptomatology or disease (Ley *et al* 1958) such antibodies arise also in rabbits injected with penicillin (Josephson 1960) In patients who give penicillin reactions the antibodies appear to be 7S and probably the antibodies are largely 19S in other persons

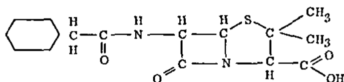
Asthma The problem of seasonal asthma differs from that of hay fever in the duration of attacks the basic reaginic mechanism being common to both Indeed progression from hay fever to asthma has been observed It has been alleged that in the asthmatic patient the tissue component can become altered pathologically through undergoing repeated allergic responses

In persons that have been termed horse asthmatics because of their responses to horse dander the hypertrophy of bronchial musculature frequently developing has been interpreted as rendering them particularly liable to respiratory symptoms and has been likened to the condition normally existing in guinea pig lung Kallos and Kallos Deffner (1937) found evidence of enlarged and emphysematous lungs in autopsy records of patients dying in asthmatic attacks and they pointed out that the special histologic character of the alterations in *Asthma bronchiale* rests on a peculiar allergic reaction of the bronchial wall and of the lung tissue (translation)

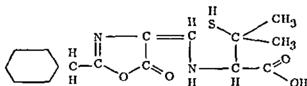
It could not be expected that prolonged

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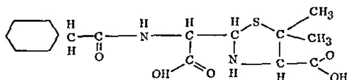
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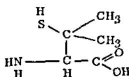
Benzylpenicillin



Benzyl penicillenic acid



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Penicillamine

Fig 5 Some reaction products of penicillin G

serum albumin (Fig 4 B) can be shown to consist of human gamma globulin and complement

Proof that the gamma globulin is antibody in these lesions is mostly wanting as is true also in systemic lupus rheumatic fever rheumatoid arthritis and periarteritis nodosa Through recent studies however a definite relationship is coming to light between acute and subacute glomerulonephritis in man and preceding streptococcal infection Antigen that appears to represent streptococcal products especially of Type XII is found to be associated with glomerular membranes in acute disease In subacute glomerulonephritis antigen is less often detected but the globulin-complement deposits in the inter mesangial spaces seemingly have the capacity to bind fluorescein labeled Type XII streptococcal antigens (Seegal Andreas Hsu and Zabriskie 1964)

Dixon (1963) has further stressed that the sites in which complexes localize and cause injury do not reflect immunologic relationship between a complex and a tissue but are selected by local factors in the tissues and the character and the amount of complexes The existence of focal tissue injuries for example may cause localization of small complexes that otherwise would be clinically ineffective

Apart from the demonstration by fluorescence microscopy of host gamma globulin in lesions a search can be made for complexes present in the circulation Ultracentrifugation actually has disclosed the presence of gamma globulin containing complexes in the sera of patients primarily those with rheumatoid arthritis that are analogous to antigen antibody complexes (Kunkel *et al* 1961) Rheumatoid factors (19S 7S) which act like antiglobulin antibody complex with the patient s own 7S gamma globulin to give products in the ranges plus 22 S and 9–17S (Williams and Kunkel 1962 view the rheumatoid factor as antibody to buried determinants on normal gamma globulin) Likewise among patients with purpura subacute bacterial endocarditis idiopathic pulmonary fibrosis macroglobulinemia and so on gamma globulin-containing complexes have become to light

Auto antibodies in Allergy The thought that antibodies might arise to an individual s own tissues and react with cytotoxic effect is strengthened by supportive observations Much evidence has accumulated especially by use of fluorescing antihuman immune globulin antibodies (cf Coons 1956) that a patient s own gamma globulin affixes in sites of specific lesions—systemic lupus erythematosus and nephrotic glomerulonephritis (Mellors Ortega and Holman 1957) subcutane

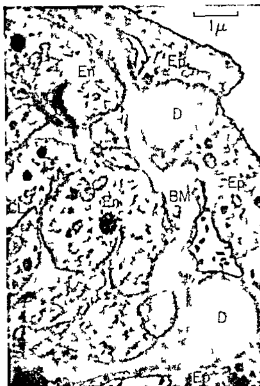


Fig 6 Portion of a glomerulus from an adult with subacute and chronic glomerulonephritis A part of one loop is visible (CL) Its lumen is filled with cytoplasm of swollen endothelial cells (En) The basement membrane (BM) is irregularly thickened and serpentine On its epithelial side are 2 dense deposits (D) resembling the deposits seen in rabbits with glomerulonephritis induced by injecting antigen over a prolonged period of time (see Fig 4) Epithelial foot processes have been replaced by sheets of cytoplasm (Ep) which cover both the deposits and the basement membrane (Drs Joseph D Feldman and F J Dixon)

asthmatic attacks would be explained by local release of preformed histamine for endogenous histamine should produce early and sharp reactions and mast cells would become exhausted A probable role of Slow Reacting Substance A' in human asthma has been predicted (cf Brocklehurst 1962 and Tables 2 and 3) since its release starts later than that of histamine and its concentration continues to mount 87 per cent of the contracting potency of lung perfusate at 30 minutes being referable to SRS-A Samples of freshly excised sensitive human lung either perfused with specific allergen or chopped and bathed in specific allergen released both histamine and SRS A exactly as did the lungs of sensitized guinea pigs or of sensitized monkeys The SRS A of both human and guinea pig origin caused contraction of human bronchioles in vitro in entire absence of tachyphylaxis hence a tissue subjected to repeated doses does not show any diminishing effectiveness A second possibility for persisting reactions in the asthmatic lung would be new formation of histamine by the inducible enzyme histidine decarboxylase (p 255) and a third possibility would be bradykinin (Tables 2 and 3), not yet demonstrated to arise in humans

Pointing once again to tissue changes in the lung tissue of asthmatics is the finding that aerosol inhalation of 0 5 per cent bradykinin reduces the vital capacity but even high doses are ineffective in nonallergic patients (Herxheimer and Stresemann 1961a)

As noted above the important finding that asthma can be produced in the *Macaca irus* monkey with human reagin offers a new tool in these studies

Physical allergies toward light (urticaria solaris) and toward cold are known Sera of such patients are found at times to permit transfer by the Prausnitz Kuestner procedure the prepared sites on the recipient being subjected respectively to irradiation with the proper wavelength of light or to chilling Such transfers show that an autologous substance must arise in the skin of all persons upon exposure only a very few persons developing hypersensitivity to it. Urticaria solaris probably may represent the oldest form of auto immune disease (Baer) The reagin is heat labile and often unstable and appears to be found in several serum fractions Thus after a reaginic serum in a case of physical allergy to cold had been separated into several protein fractions successful transfer was possible only by use of a mixture of component fractions (Sherman and Seebohm 1950) In reaginic type cold allergy Lennart and Shelley (1961) showed by punch biopsy that tissue mast cells underwent degranulation in chilled areas of skin releasing histamine

Among persons showing urticaria to light the reaginic factor is found chiefly in persons sensitive to the 2 800–3,200 A range of ultra violet and having an atopic background and reagins have been found also in certain but not all persons sensitive to the 4 000–5 000 A range of blue and violet light

Not all instances of urticaria due to light are allergic in nature one type seems to correlate with a heightened concentration of protoporphyrin IX in the skin, and some examples are referable to *phototoxicity* of ingested drugs such as sulfonamides and demethylchlortetracycline (Declomycin) (Epstein 1939, Harber *et al* 1961) But certain drugs and chemical compounds have proved to be *photo-allergic* in some individuals—the antihistamine promethazine (Phenergan) chlorothiazide (Diuril) sulfanilamide

Tissue Injury Studies on experimental animals already described (p 259) provide the current model for exploring tissue injury as a consequence of antigen antibody complexes Most often involved probably is the poorer antibody producer in whom antigen is not subjected to prompt immune elimination permitting soluble antigen antibody complexes to form and to persist It will be recalled that complexes can persist for long periods of time sequestered in the thickened basement membranes of the kidney glomerulus without causing acute inflammatory reactions Dixon points to concomitant persistence of associated renal malfunction lasting as long as a year i e , proteinuria (Dixon 1963)

An example of human subacute and chronic glomerulonephritis is shown in Figure 6 The segregated dense deposits resembling in location and size those arising in rabbits injected for months with bovine

antibodies can be involved—being either complete incomplete or hemolytic (with complement) and others are active between 4 to 16° or more being macro globulins (19S) of the cold variety utiliz ing complement In patients that recover owing to treatment the antibodies disappear

The origin and the significance (in terms of allergic episodes) of certain other impor tant factors found in the serum of patients have not yet been determined Patients with lupus erythematosus (L E) possess a gamma globulin which is responsible for the forma tion of the bizarre L E cell that appears in the blood the factor fixing to the nuclei (Miescher *et al* 1954 Holman and Kunkel 1957) of polymorphonuclear leukocytes which then form a swollen body become free and undergo ingestion by normal polymor phonuclear leukocytes The factor fixes to the separated nuclei of several species and is viewed as an auto antibody to nucleoprotein or deoxyribosenucleic acid (cf Robbins *et al* 1957)

The idea has been voiced that some allergic diseases may occur without participation of any antigen Thus if molecular changes should arise in gamma globulin that would allow these molecules to aggregate comple ment fixation could take place resulting in nonspecific agglutination of red cells and platelets in the absence of antigen and anti body (Ishizaka *et al* 1961 1962)

To study auto immune diseases various laboratory models have been used many using antigens that differ from those of the subject animal itself Often denatured autol ogous proteins are used as antigen or nor mal tissue is injected with mycobacterial paraffin oil adjuvant or guinea pigs whose tissues contain Forssman antigen are injected with anti Forssman immune globulin Hal pern *et al* (1963) have injected papain re peatedly into monkeys and rabbits leading to destructive alterations in the tissues and a finding of antibodies toward tissue structures containing glucoproteins In one model a graft versus host reaction (p 289) is estab lished by appropriate cellular transfer and the pathologic events occurring in runt dis ease are noted The value of such study in volving erythema purpura acanthosis and various extreme lesions as well as the histo logic resemblances to lupus erythematosus and scleroderma has been stressed by Stastny *et al* (1963)

ALLERGIC INFLAMMATION DELAYED RESPONSES

Allergic reactions that require some hours to become manifest after the test material is applied are placed in a separate category not because of the delay in development but rather because they exhibit no readily de monstrable relation to circulating antibodies and the procedure of passive transfer by means of serum is typically unsuccessful Transfer with white cells in contrast has been demonstrated in several key instances without direct evidence of circulating im munoglobulin (Table 1) The coexistence of a delayed type of response and a circu lating antibody has been noted in some cases which is not surprising since the stimuli giv ing rise to delayed type hypersensitivity ex cites the antibody forming apparatus as well even if not in an equal degree

Dermal sensitivity is induced chiefly by percutaneous absorption following contact of the skin with substances of low molecular weight (simple chemical substances nickel salts urushiol from the poison ivy plant and the like) or with products of fungi growing superficially in the skin Upon a subsequent exposure to the same agent there appear reactions of eczematous type such as hy peremia macules papules or even vesicles as the epidermis separates and fluid accumu lates beneath itching can occur as well His tologically there is infiltration with lympho cytes and monocytes giving rise to indura tion as contrasted with the edema seen in immediate type reactions

Invasion of the body by a variety of infec tive agents (bacteria fungi parasites viruses etc) establishes hypersensitivity around foci of infection The presence of hypersensitivity is detected by injecting various extractives or metabolites of the infectious agent (or entire viral suspensions) into the skin (cf Jadassohn 1932) with resulting cellular in filtration hyperemia and gradually increas ing induration

ous nodules of rhematoid arthritis and heart valves in fatal rheumatic fever (Vazquez and Dixon 1956)—and often there is no known alteration in the tissues *to suggest the reason* for auto antibody formation

It is first necessary to set apart instances in which the antigenic stimulus is provided *from without the body* as in the production of thrombocytopenic purpura by the administration of allergenic drugs

In studying thrombocytopenic purpura that followed the ingestion of the drug Sedormid Ackroyd (1949 1955) found an antibody that agglutinated and evidently lysed the patient's platelets whenever the drug was ingested In vitro studies on the resulting antibody drug platelet complex have been possible in both Sedormid and quinidine hypersensitivities (Ackroyd 1955 Dausset 1957 Bolton 1956 Shulman 1958) The antibody fixes complement It attaches so loosely to the platelet that is freed by dialyzing away Sedormid

The idea has been advanced that group A streptococci contain an antigen immunologically related to human heart tissue and may give rise to antibodies that function as organ specific auto antibodies (Kaplan, 1962 cf Cavelti 1947) this could perhaps explain the finding of antibodies fixed in the heart tissues of persons dying of acute rheumatic fever

In other areas the alteration of tissues by disease or mechanical trauma could lead to the formation of nonself' products of potential antigenicity

Thus *Schwentker and Rivers (1934)* showed that monkey brain—usually altered by autolysis or viral infection—was antigenic in monkeys their work led directly to experiments that resulted in production of iso allergic encephalomyelitis for which a highly artificial adjuvant system is now employed (this disease will be discussed later) The same device—the use of dead mycobacteria *in paraffin* oil and tissue maintained in water droplets for slow release—leads to autosensitization vs thyroid (Witebsky and Rose 1956) testis (Voisin *et al* 1951 Freund *et al* 1953) and so on In all of these states antibody is demonstrable but does not appear to play a chief role

Often however there is no clue as to the 'antigen' that gives rise to auto antibody For example in idiopathic thrombocytopenic *purpura (ITP)* a plasma factor has been demonstrated leading to a marked and prompt reduction in the numbers of platelets in transfused normal volunteers and producing frankly hemorrhagic manifestations in some of them (cf Harrington Minnich Hollingsworth and Moore 1951 Harrington Minnich and Arimura, 1956) the activity is found to reside in the globulin fraction (Evans *et al* 1951) Yet there is no good in vitro technic for studying the ITP plasma factor such as that which exists for the anti platelet antibodies induced by drugs

Indeed the view that antigen should be some altered body constituent capable of inciting antibody production has been doubted The alternate theoretical view—by no means proved—is that special cell lines having potential antibody forming capacity remain within exceptional individuals under some sort of control (tolerance is alleged) but eventually come to react to say the individual s erythrocytes and form antibody against entirely normal cells This explanation is advanced to explain the fate of a particular strain of mouse the New Zealand Black in which all animals eventually develop auto immune hemolytic anemia some showing lupus nephritis also (Bielschowsky Helyer and Howie 1959 Helyer and Howie 1963) In this disease in which antibodies attaching to the red cells come to be formed by splenic cells between 4 and 12 months of age (cf Holmes Gorrie and Burnet 1961) outbreeding was seen to alter the age of onset and made the predominant feature sometimes lupus nephritis with renal failure sometimes hemolytic anemia In man too attention is being paid increasingly to susceptibilities within family groups—for example toward auto immune thyroiditis

Auto immune hemolytic anemia of man is a disease seemingly having no intimate relevance to hypersensitivity globulins become adsorbed to erythrocytes, resulting in early removal and anemia and often ending in death The antibodies encountered among patients fall into various types Some are active at 37°—both γ globulin and non-γ Rh

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The 'delay' in onset of reactions both in testing for contact dermatitis and for tuberculin type allergies appears to represent the time required for white cells to leave the vessels accumulate at the test site and react there with the test material There seems to be no inherent sensitiveness of the skin (Frei 1928 Landsteiner *et al*, 1948 Livingood 1952 Curtis 1952) Indeed in transplanting skin between twins one of whom had been sensitized to dinitrochlorobenzene Haxthausen (1943) observed that skin of the normal brother exhibited hypersensitivity after being grafted to the sensitive twin and, conversely that a skin graft from the sensitized brother to the normal twin showed no capacity to react

Further discussion of mechanisms will be given below Here it is useful only to note that the term cell bound antibody has unfortunately arisen conjuring up the picture of lymphocyte or monocyte carrying preformed antibody firmly bound to it There is little evidence that this is the situation according to one recent report continued synthetic function of the competent cell may be required to effect reaction with allergen (Bloom *et al* 1964)

It would appear from the several aspects of delayed hypersensitivity (which too often are studied singly) that allergic conversion is associated with (1) an altered behavior of lymphoid cells (2) an altered behavior of macrophages and (3) often a stimulation to form immunoglobulins Without doubt future studies will put these into sharper focus

The hypersensitive state related to infectious processes will be considered first The outstanding example of reactions of delayed type was provided by Koch s discovery in 1891 of the tuberculin tuberculosis relationship although the underlying allergic basis was not recognized until 1903 (von Pirquet) Owing to several confusing factors a clear distinction between the tuberculin reaction and reactions of the anaphylactic type was drawn only much later (Coca 1920 Calmette 1920 Zinsser 1921)

Tuberculin Hypersensitivity

In Koch s experiments tuberculous guinea pigs were found to possess a special reactivity when superinfection was attempted At the subcutaneous injection site there occurred a massive inflammatory reaction of the tissues, walling it off within 3 days and usually leading to slough—the Koch phenomenon ' Since living cells were not necessary, Koch sought a bacterial extract possessing the same property and attained it by heating the mycobacteria for some hours in the medium in which they had grown (meanwhile concentrating the fluid), and finally removing the bacterial residue Injection of this preparation called O T or old tuberculin caused a severe to lethal shock of delayed type termed the systemic reaction A *local* reaction occurred not only as a papule in the skin where the injection had been made but also along the needle track and the draining lymph nodes were discolored and enlarged There were also *focal* reactions of inflammation around existing tuberculous lesions with dense leukocytic infiltrations and with hemorrhagic exudate in serous cavities The focal reactions seem to give rise to the general toxemia and death

In 1903 von Pirquet advanced the hypothesis that tuberculin shock was another of the phenomena of sensitization and this belief led him to the discovery in 1907 of the *cutaneous tuberculin reaction,* so important diagnostically A delayed type of *ocular reactivity* was noted almost simultaneously Thus the relatively avascular cornea of tuberculous guinea pigs reacts to direct injection of tuberculin (Holley 1935 Rich and Follis 1940) with edema swelling of the fibers infiltration with granulocytes and necrotization of corneal cells

Indeed the 4 types of response—local focal ocular and systemic—remain as our most stringent criteria of delayed type hypersensitivity to water soluble allergens but such critical tests are possible only in sensitized laboratory animals *local* reactions at the site of skin tests showing induration at 48 hours *systemic* reaction to injections made by the peritoneal or intravenous routes, and *corneal* reactions resulting from intracorneal injection Even in diagnostic testing by intracutaneous injection (Mantoux) it is necessary to select the dosage of tuberculin with caution for an excess of tuberculin may cause not only unduly severe reactions in the skin but also some degree of lighting up

around tuberculous lesions and febrile systemic reactions (Tytler 1930)

Obviously bacteria offer numerous antigens to the invaded host In the case of *Myco tuberculosis* circulating antibodies to perhaps 7 of these have been discovered by the application of special technics (Boyden and Sorkin 1956) although the concentration of the antibodies is seldom high Indeed one of the early difficulties in interpreting the tuberculin reaction had to do with the occasional demonstration of an anaphylactic state in tuberculous guinea pigs It is now rather well agreed that these antibodies are not causally related to the delayed type hypersensitivity Several of the tuberculoproteins tested in their native state elicit cutaneous delayed type reactions one being heat-coagulable and of large molecular size (ca 44 000) For the purpose of human skin testing it would seem to be desirable to elect low molecular and even heat altered material as long as it is serologically reactive in order to avoid the acquisition of sensitiveness to proteins through repeated skin testing Present practice however is to prepare the testing materials such as PPD (purified protein derivative) from culture filtrates that approach the native state

When suitably diluted tuberculin is injected into the skin of the tuberculous individual redness appears after some hours and the local inflammation with its associated infiltrate gradually increases in intensity and extent for from 15 to 48 hours and attains a typical firm induration when the dose and the degree of sensitivity cause a more severe reaction there are central blanching and a gradually developing innermost livid zone which often becomes necrotic The inflammation slowly fades but the lesion is palpable for some days and pigmentation may be seen for several weeks

Histologically the reaction to tuberculin is attended by characteristic focal accumulations of mononuclear cells (Dienes and Mallory 1932 Laporte 1934 Fisher to be published) In tuberculous guinea pigs Fisher found that lymphoid cells left the vessels in the corium and migrated upwards toward the epidermis and the hair bulbs Within 12 hours the epidermis became acanthotic and the lymphoid cells formed focal accumulations (spongiosis) with vesiculation and perivascular granulomas appeared in the corium Hyperplasia occurred in the papillary cells of the hair roots along with destruction of the hair bulbs causing loss of hair (When sensitization is effected by using dead mycobacteria in paraffin oil other features are noted in addition probably owing to participation of immunoglobulins) Studies of tuberculin reactions in rabbits by Gell and Hinde (1951 1954) showed proliferation and differentiation of the infiltrating monocytes and also of local histiocytes and mesenchymal cells From their studies with rabbits sensitized with dead mycobacteria and hydrocarbon—this species producing antibody in high concentration—these workers were less inclined than others to distinguish between Arthus and purely tuberculin reactions Indeed when necrosis results from tuberculin reactions there are no differentiating histologic features between severe tuberculin and Arthus reactions

By injection of killed mycobacterial cells it is possible to induce only low grade sensitivity to tuberculin but there is remarkable enhancement of sensitization when the dead bacilli are suspended in hydrocarbons such as paraffin oil Many attempts had been made to obtain a bacillary extract capable of substituting for the whole killed cells in inducing the delayed type of hypersensitivity Finally Raffel found a wax fraction of the cells in conjunction with tuberculoprotein present as an impurity or intentionally admixed to possess the property sought (cf Myrvik and Weiser 1952) The directive effect of the wax in inducing sensitization to tuberculin appears to be dependent on its lipopolysaccharide Choucroun (1939 1947) had separated a lipopolysaccharide that gives rise to delayed type hypersensitivity while Asselineau and Lederer (1949) extracted wax D from human tubercle bacilli its counterpart in bovine strains remaining bound to the cell walls Wax D both sensitizes to tuberculin and possesses the adjuvant property of mycobacteria It is a peptidoglycolipid containing mycolic acid joined in ester linkage to a polysaccharide composed of arabinose mannose galactose glucosamine and galactosamine and through the hexosamine to a heptapeptide containing

alanine glutamic acid glycine and α, ϵ diaminopimelic acid (Asselineau, Buc Jolles and Lederer 1958)

After a single large injection of tuberculin has produced severe constitutional reactions desensitization—usually incomplete—is found in tuberculous animals and human beings Desensitization is peculiarly hazardous because of the danger of focal reactions but desensitization by repeated gradually increasing doses of tuberculoprotein has often been carried to a point at which the patients were able to tolerate without a focal or constitutional reaction, doses of the protein which in the undesensitized body would undoubtedly have produced extreme focal reactions and death (Rich 1951) The desensitization of tuberculous guinea pigs is a difficult task requiring large repeated doses of the tuberculin preparation PPD, but during the treatments the skin is maintained truly nonreactive and it even will react to intradermal spread of dyes in the same way as does normal skin (Birkhaug and Berle 1945) However, sensitivity returns when administration is discontinued

Cellular sensitivity to tuberculin was observed by Rich and Lewis (1928 1932) it was evidenced by a decided inhibition of migration of the allergic cells from the explant into the surrounding tuberculin containing plasma and by the fact that the relatively few cells which do wander out die in a few hours Confirmation has been forthcoming when proper concentrations of tuberculin are used but cells of well sensitized individuals are required (cf Aronson, 1931 Moen and Swift 1936, Heilman 1944 Waksman 1953 Florio *et al* 1958 Hall and Scherago 1957) Apparently sensitivity of macrophages is evident even in their descendant or daughter cells at least of the immediately succeeding generations (Moen 1936) and sensitivity of fibroblasts is variable but often trivial It is not surprising in view of failure to find inherent sensitiveness of the skin that sensitivity is not seen in cultures of corneal epithelial cells or cutaneous or hepatic epithelium (cf May and Weiser 1956)

Yet not until 1945 was it recognized that living white cells of tuberculin sensitive animals serve to transfer the delayed type of reaction to another animal of the same species (Chase, 1945, Cummings *et al*, 1947, Stavitsky 1948) Competent cells contained in peritoneal exudates lymph nodes spleens or blood would induce hypersensitivity to tuberculin for 5 to 9 days termination appeared to depend on homograft type rejection of the transferred cells for transfers between isologous guinea pigs were durable (Chase, 1960 1963 Bauer and Stone, 1961) Further, Kirchheimer and Weiser (1947) showed that systemic reactivity to tuberculin is transferable likewise by means of cells (cf Kirchheimer *et al* 1949, Wesslen 1952) It is highly probable that analogous transfers were encountered in older attempts since tissue and white cells were often included inadvertently in the material transferred (cf Bail 1910 Fellner, 1919), but the experiments were discounted because they lacked reproducibility

When transfer is made by injecting large numbers of competent cells intradermally into normal recipients special observations have been possible If rather large amounts of tuberculin were given parenterally tuberculin reactions (probably with participation of a Shwartzman effect) occurred in the sites of cellular deposition in both guinea pigs and rabbits (Metaxas and Metaxas Buhler 1948, 1949 Wesslen 1952) It was shown that mixtures of cells and tuberculin were fully effective when injected intradermally (Metaxas and Metaxas Buhler 1955) By means of the latter technic mixtures of tuberculin and competent rat cells reportedly have given effective reactions in guinea pig recipients a first instance of crossing the species barrier (Bacon Dabney and Wallace 1961 cf Wallace, 1958)

Under normal conditions of cellular transfer, in which cells must survive and escape from blood vessels to the test site no large number of donor cells (labeled proportionately by tritiated thymidine) is found to participate The processes leading to cellular damage in the test site are largely unknown but will be discussed below

More recently, intensive study has begun on the direct reaction in vitro between competent cells and tuberculin Cellular migration from explants of macrophages taken up to 10 months following sensitization was

inhibited consistently in tests with 10 mcg /ml tuberculin PPD and sensitivity was seen to spread gradually from cells of the nearest lymph nodes to splenic cells and hepatic macrophages (Carpenter 1963) Using macrophages and lymphocytes packed in capillary tubes David *et al* (1964) studied cellular outgrowth into culture medium containing PPD Not only were sensitive cells specifically inhibited but all migration was blocked provided that as little as 2 5 per cent of tuberculin sensitive cells were mixed with normal cells

A phenomenon of lympholysis was de scribed by Favour (1947) When tuberculin was mixed with lymphocytes from tubercu lous mice a loss of 20 to 40 per cent of these cells occurred in less than 1 hour The effect appears to depend on an antibody present in tuberculous plasma and serum comple ment is required for lysis In guinea pig and man granulocytes and large mononuclear cells are lysed also but more slowly The serum factor has been stated to be released by the tuberculin sensitive lymphocytes upon incubation in normal plasma (Miller and Favour 1951) In studies by Waksman (1953) and Waksman and Bocking (1954) inhibition of macrophages in tissue culture and not lympholysis paralleled dermal re activity to tuberculin (cf Waksman s exten sive review 1958)

A wholly different phase of sensitization—a slow synthesis of gamma globulin—has been emphasized by studies of lymphocytes in culture following the addition of allergen As Pearmain Lycette and Fitzgerald (1963) showed with tuberculin and is now well con firmed (cf Hirschhorn *et al* 1963a b) lymphocytes of persons with delayed type hypersensitivities when exposed to the allergen in maintenance culture are found to respond with significant changes in 5 days they aggregate increase in size and synthesize gamma globulin show mitotic figures (0 6 to 3%) and blast cell formation (3 to 20%) and some cells come to resemble plasmocytes For undiscovered reasons normal lymphocyte cultures are stimulated to undergo this same sequence of changes starting within 4 hours and completed in 3 days upon addition of a leuko agglutinin present in crude phytohemagglutinin or lectin Nearly every cell responds to leuko agglutinin but in the case of hypersensitive donors only a proportion of the total cells reacts to specific allergen (5 to 35%) prob ably those that are immunologically com petent cells The observation may well bear on the acquired capacity for antibody pro duction that follows the onset of delayed hypersensitivity Boyden and Suter (1952) showed for example that in tuberculous guinea pigs cutaneous testing with tuberculin stimulates production of circulating antibody within 5 days Whatever the relation to de layed type hypersensitivity may prove to be the ability to discover the causal allergen should make this technic a powerful tool

Quite novel aspects of tuberculin hyper sensitivity diverging in several respects from the above account have been forthcoming from studies in man chiefly by Lawrence By cellular transfer of small volumes of white cells from the peripheral blood Lawrence reported transfer in man of tuberculin (1949) and of streptococcal (1952) hyper sensitivities Lawrence then found (1954 1959 1960) that lysates of the cells induced sensitization which was unaffected by ex posure to deoxyribonuclease ribonuclease or trypsin Because the induced sensitivity was found to persist for many months and successive serial transfers were possible using recipients cells lyzed in turn the concept of a replicating transfer factor was de veloped to explain the retained sensitivity in later cell generations Transfer factor is liberated when cells are incubated with tuber culin no evident damage occurring to the cells and it is readily separable from DNAse treated cells by passage through dialysis membranes The active material has a molec ular weight of 10 000 or less and is un affected by ribonuclease it is not a protein (Lawrence *et al* 1963) The subject now is opened to identification of the active ma terial and an understanding of the mechanism by which it operates

If as has been stated transfer factor is unique for the human being human mono cytes still react to tuberculin as explants the points at issue concern (1) replication in vivo (2) the existence of a preformed dis sociable factor bound to lymphocytes (3) the apparent loss by living lymphocytes of

alanine glutamic acid, glycine and α ε-diaminopimelic acid (Asselineau Buc Jollès and Lederer 1958)

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Delayed type hypersensitivity can be estab lished in rabbits with *viridans* streptococci by intracutaneous injection and by several other routes but not by intravenous injection Apparently in connection with the development of some focal tissue reaction the animals presented cutaneous ophthalmic and systemic hypersensitivity to the admin istration of living streptococci all being ex pressions of a delayed type of reaction un related to the amount or the type of circulating antibody and not transferable by serum

To relate further the mechanisms of strep tococcal and tuberculin hypersensitiveness Moen (1936a) examined both sorts of sensi tivity in parallel in tissue culture using ex plants of tuberculous tissues and explants of the spleen of guinea pigs infected with a natu ral streptococcal pathogen (group C hemo lytic streptococci from guinea pig lymph adenitis) The presence of streptococcal extract led to a slowly developing specific toxic effect on the cells of streptococcus infected animals quite similar to but quan titatively less than that of tuberculin on tuberculin sensitive cultures The strepto coccus sensitive cells exhibited more unequal gradations of sensitivity than were seen in tuberculin sensitive cultures some cells being killed rapidly others being only slightly in hibited Very much less evidence of persisting sensitivity was seen in subcultures of the streptococcus sensitive cells as compared with explants of tissues from tuberculous animals

As mentioned above Lawrence (1952) has reported transfer of streptococcal hyper sensitivity from man to man by means of peripheral blood leukocytes

Pneumococcal Allergy Pneumococcal allergy has been studied in much the same way as has streptococcal hypersensitivity Deposition of vaccine in the skin of rabbits leads to a delayed type of skin reactivity to ward pneumococcal vaccine or nucleoprotein and a delayed type of ocular reactivity (Julianelle 1930 Harley 1935 1937) the manifestation of reactivity to nucleoprotein is passively transferable by means of serum and apparently is of Arthus type whereas neither the allergy to vaccine nor the eye reaction is so transferable In contrast the injection of intact cocci by the intravenous route leads to the development of anticarbo hydrate antibodies and to immediate type reactivity expressed toward the correspond ing polysaccharide

During the course of pneumococcal pneu monia 2 types of specific skin reaction were observed (Tillett and Francis 1929) one being in response to the type specific capsular polysaccharide and appearing at the onset of convalescence consisting of an immediate reaction of urticarial type and the other elicited by species specific nucleoprotein and being of the tuberculin type A 3rd type of reaction can be produced by intradermal tests with somatic C polysaccharide of pneumococci during a limited period of the disease the reaction depends on a special protein (termed the C reactive protein) which appears as a consequence of many acute infections not only with the pneumo coccus and happens to react with C poly saccharide even in vitro An analogous factor not capable of reacting directly with C polysaccharide appears also in animals during acute inflammatory responses viz to typhoid vaccine (Kushner and Kaplan 1961)

SKIN TESTS

To make any test practicable for diagnosis it is necessary to use relatively purified preparations to know the proper doses with respect both to tissue irritation and concen trations that will not cause focal reactions in positive reactors and to understand the specificity of the reaction It must also be known whether the material employed for skin testing can lead to active sensitization and vitiate future tests Even in the most

their property of effecting transfer after transfer factor has been dissociated (4) the relation of the small nonprotein molecule to synthetic functions awakened by immunologic stimulation and (5) the functional specificity of transfer factor Transfer factor appears not to have been encountered in animals (Bloom 1963) previous reports seemingly being explained by technics of testing the animals

Allergy in Microbial Diseases Other Than Tuberculosis

In other microbial infections (with bacteria fungi viruses) and in certain parasitic infestations there is exhibited much the same sort of delayed type reactions when the corresponding agents or extracts thereof are put into the skin In variable degree one finds also the other chief attributes of delayed type sensitization—systemic reactions sensitivity of the cells in tissue culture toward the respective allergens and characteristic damaging reactions upon intracorneal injection However many reactions are less pronounced than in the tuberculin tuberculosis relationship for as Boyd has remarked tuberculin allergy is one of the more extreme examples of bacterial allergy Furthermore one finds the early type of sensitivity far more frequently the extracts employed for testing sometimes giving immediate type reactions particularly in relation to a content of specific polysaccharides Often both types of sensitization coexist probably related to different constituents of the test material

The degree to which microbial allergies may shape the disease pattern has been long debated Fundamental studies regarding the effect of delayed hypersensitivity on invading microorganisms were made in mice by Mackaness (1962) When *Listeria monocytogenes* selected for virulence and multiplication within macrophages was injected in sublethal numbers intracellular multiplication progressed to the 4th day and then ceased abruptly Delayed type hypersensitivity appeared at this time demonstrable by the reactivity of the footpad to culture filtrate and coincidentally macrophages showed accelerated mitotic activity destruction of bacteria and resistance to superinfection Similarly intense mitotic activity was caused in the peritoneal macrophages of hypersensitive mice when culture filtrate was injected Seemingly the altered macrophages accounted for the resistance of convalescent mice to challenge Resistance continued for a few weeks and then gradually diminished even though hypersensitivity persisted As long as hypersensitivity was present there was capacity to give an accelerated response to reinfection perhaps through formation of a new population from surviving hypersensitive macrophages In general however, it is not possible to isolate so clearly the processes ensuing after invasion by microorganisms and the role played by hypersensitivity usually has remained indeterminate In the same way a greater destruction of tubercle bacilli occurs in vitro within macrophages of hypersensitive animals than within normal macrophages (cf review by Elberg 1960 Suter 1953 1954 Fong *et al* 1957 1963) but the role played by white cells in delayed type hypersensitivity probably exceeds that of intracellular digestion by monocytes It may be noted that immunity to tularemia appears to have been passively transferred briefly by spleen cells and peritoneal leukocytes (Allen 1962)

Streptococcal Allergy Evidence of infection with streptococci usually is sought in the form of circulating antibodies directed against streptococcal enzymes and type specific antigens However preceding infection can be witnessed by delayed type allergy toward streptococci Relatively large samples of the population have been tested by means of killed streptococcal cells (vaccines) cellular substances (nucleoprotein M substance) and culture filtrates Mackenzie and Hangar (1927) found the incidence of sensitivity to increase gradually with age and superimposed upon this finding a sharp rise in the number of reactors whenever diseases associated with streptococci became prevalent Lawrence (1952) in testing 472 persons having no apparent streptococcal infection found that only about 11 per cent failed to react at all but that only half of the subjects exhibited a well-established degree of sensitivity The sensitivity expressed was unequal to his testing materials suggesting elective sensitization to different bacillary constituents —vaccine cellular M substance and a par

tially purified culture filtrate rich in the enzymes streptokinase and streptodornase

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Skin Tests

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studied case tuberculosis investigation does not cease In instances in which both early and delayed reactions can be shown it is important to evaluate independently the significance of each type of reaction

In general when the method of testing is specific such tests have a significance similar to that of tuberculin a negative reaction in a healthy subject allays suspicion and a positive reaction denotes the occurrence of infection but gives no generally reliable indication of current activity A reaction of unusual size will require close scrutiny It is most informative when the skin reactivity is known to have been acquired recently One must recall furthermore that in advanced stages of all diseases including tuberculosis the skin may fail to react this condition is called anergy and has been considered to be a consequence of an exhaustion on the part of the tissue cells Also in intercurrent infection such as measles and chickenpox the tuberculous host temporarily may cease to give skin reactions to tuberculin Even a positive test which is specific may not always be of practical diagnostic value for example Sulzberger (1940) points out that interpretation of reactions to extract of Monilia (Oidiomycin) must be weighed against the well nigh universal exposure to various species of Monilia

In special cases delayed type hypersensitivity to proteins is encountered such as tetanus toxoid and diphtheria toxoid Consequently in performing the Schick test with diphtheria toxin—a test for the presence of neutralizing antibodies that avert the normal toxicity of the material for skin—a parallel test with heated toxin or with toxoid is used to detect the existence of sensitivity to the protein itself This tuberculin type of response representing the so-called false or pseudopositive Schick reaction lasts a day or so and then fades usually it can be differentiated from the positive Schick reaction in that *the true toxic effect appears slowly and* develops increasingly during the course of 4 or five days Both to purified diphtheria toxin and toxoid the tuberculin type of sensitivity occurs in about one fifth of young adults infrequently in children (Pappenheimer 1948) (In addition immediate type flare and wheal reactions occur in some individuals to these same constituents of diphtheria toxin and toxoid)

Tuberculosis Of the several varieties of tuberculoproteins and other mycobacterial products the preparation PPD is chiefly used in this country The 3 test strengths contain in 0 1 ml doses 0 02 0 2 and 5 0 mcg (1, 10 and 250 TU or international tuberculin units) termed respectively first test strength, intermediate strength and second test strength Other preparations of Seibert s from human tubercle bacilli are being evaluated currently some apparently being more reactive than standard PPD S (Vakilzadeh *et al*, 1962) (It should be mentioned that intradermal testing with a suspension of Choucroun s lipopolysaccharide PmKo gives more information at certain stages of the disease than does PPD see Kourilsky Choucroun and Gresland 1963) With the realization that persons reactive only to 250 TU of PPD S come from certain geographic areas (Edwards *et al* 1959) study has shown that tissue invasion in those localities occurs by strains of the atypical mycobacteria Tests made with PPD preparations of atypical mycobacterial strains place particular importance on PPD B prepared from the Battey (Group III) stain and to *PPD G* prepared from a scotochromogen (Group II) strain (reviewed by Youmans, 1963) Such tuberculins in standard doses of 5 TU (1 mcg) are especially useful in revealing infections with the atypical mycobacteria Avian PPD gives reactions in these particular patients also

Leprosy Lepromin an extract of heated human lepromatous nodules rich in bacillary bodies provokes skin reactions in patients with tuberculoid leprosy (cf Mitsuda 1936) (Lepromin produces cross reactions in tuberculin sensitive individuals) In addition to the initial tuberculin type reaction appearing within 2 days and termed the *Fernandez reaction the presence* of bacillary bodies can provoke nodular reactions starting by the 7th day and developing progressively in size for a month (Mitsuda reaction) It is interesting to note that the selective reactivity of lepromin for patients with tuberculoid but not lepromatous leprosy has

allowed positive identification of isolates growing in animal tissues as true *M leprae* (Shepard and Guinto 1963)

Brucellosis Skin reactions have been employed to detect present or past infection with Brucella both with Brucella vaccine and with an actively antigenic nucleoprotein extract Brucellergen (Huddleson 1943) The skin reactivity apearing in brucellosis of the guinea pig has been transferred by means of white cells (Metaxas Buhler 1952)

Tularemia Chemically treated (nitrous acid) vaccines have been injected intradermally as a diagnostic tool (Foshay 1940) During this disease as in pneumococcal pneumonia intradermal injection of *P tularensis* polysaccharide gives an immediate reaction of urticarial type Later when the patient s tissues contain an excess of polysaccharide intradermal tests with specific goat antipolysaccharide also produce an immediate type of reaction

Glanders The injection of *mallein* prepared from the causative agent of this disease *Actinobacillus mallei* can cause local focal systemic and ocular reactions as described for tuberculosis Cutaneous testing of infected horses and man has been practiced as well as of experimentally infected guinea pigs

Paratuberculosis of Cattle (Johne s Disease) The testing material is johnin a preparation made like tuberculin from the etiologic agent *Myco paratuberculosis* and variously purified dermal systemic and conjunctional reactions are encountered

Allergic Reactions to Fungal Materials Skin test materials are available to test for present or past infections with histoplasmosis coccidioidomycosis and blastomycosis all prepared as filtrates of culture medium (synthetic) which has supported growth of the fungi for several months in the mycelial phase In general tests for hypersensitivity are performed and interpreted as with the tuberculin tests Readings should be made both at 24 and 48 hours since significant reactions may vanish after the 1st day (Fiese 1958 Salvin 1959) In fungal diseases cutaneous anergy may develop as the lesions become widespread. The test materials—histoplasmin coccidioidin and blastomycin—are adjusted in potency to match reference standards and are diluted 1 100 for use in testing coccidioidin is diluted 1 1 000 for persons known to be highly sensitive

The active components in the skin test materials that produce delayed skin reactions are viewed as nitrogen-containing polysaccharides reportedly free of protein although one product prepared by Salvin and Smith (1959) was a protein-carbohydrate complex (Edwards Knight and Marcus 1961 see review by Salvin 1963) Polysaccharide seems to be the active principle in sporotrichin and paracoccidioidin also

The 3 test materials give varying degrees of cross reactions There is a particularly high degree of cross reactivity between histoplasmin and blastomycin and attempts have been made to eliminate the problem by extracting specific fractions Thus Dyson and Evans (1955) have suggested as a more specific test substance an alternative preparation separated by fractional alcoholic precipitation from culture supernatant of the yeast phase of *Blastomyces dermatitidis* Coccidioidin is more specific for reactions to it are found less frequently in histoplasmosis and blastomycotic infections than reactions with histoplasmin in coccidioidal infections (Smith *et al* 1949)

By reason of skin test surveys Edwards and Palmer (1963) hypothesize that in certain geographic areas that are free of coccidioidomycosis (e g Louisiana) the large number of feeble reactions encountered to histoplasmin probably represents cross reactivity reflecting infection with some new agent possibly another fungal agent

As in microbial hypersensitivity inhibition of macrophage migration—partial but specific—has been noted when histoplasmin was added to splenic explants of animals sensitized with *H capsulatum* (Carpenter 1963)

The coccidioidin hypersensitivity system has been studied recently for purposes of transfer of hypersensitivity (*vide infra*)

Allergic Reactions to Viral Materials Evidence of hypersensitiveness of the delayed type was observed unknowingly by Jenner in 1798 with the virus of cowpox individuals

rendered immune by inoculation with cowpox virus later responded to this or the closely related smallpox virus with a small local redness that appeared within 48 to 72 hours and then faded quickly This manner of response was interpreted as an allergy by von Pirquet and Schick in 1903 the role of hypersensitivity was convincingly demonstrated when Hooker (1929) injected heat inactivated virus into the skin of cowpox immunized individuals

The Frei test (originally an injection of diluted and heat sterilized pus from the lesion later of infected mouse brain) is now performed with antigen obtained from chick-embryo cultures A skin test betokening past infection with mumps virus has been developed by Enders *et al* (1945 1946) for the assessment of immunity Newer test preparations have been made of viral material grown in the allantoic fluid of embryonated eggs (Henle *et al* 1951)

Allergy in Parasitic Infestations In contrast with the above observations the form of allergic response that has received chief consideration in parasitic helminth infestations—for example schistosomiasis echinococcus disease filariasis trichiniasis and ascaris infestation—has been the immediate reactions of the wheal and-erythema type found when the skin of the host is tested with extracts of the body substance of the same or related parasites usually antibodies can be demonstrated in the serum by serologic methods at the same time Asthma and rhinitis are sometimes elicited upon exposure to the specific agents While there is often an early reaction to hydatid fluid in echinococcus infestation it appears that the delayed type or *Casoni reaction* should be given chief diagnostic interpretation

In protozoan infestations skin tests have been less useful The only instance to be mentioned is leishmaniasis in which a tuberculin like reaction has been observed in skin tests made with an extract of cultured Leishmania

Even as an aftermath of sensitization by insect bites delayed allergic reactions have been demonstrated (Benson 1936) independently of early reactions (and corresponding reagins) which occur at times both varieties may be seen in the same individual

THE ADJUVANT EFFECT OF TUBERCLE BACILLI AUTO-ALLERGIC DISEASES

Special properties of *Myco tuberculosis* came to light when Dienes (1929) showed that the injection of protein antigens into a tuberculous focus led to a delayed type of hypersensitivity directed against the protein as well as the expected appearance of circulating antibody The induced tuberculin type hypersensitiveness to egg protein as Burnet (1948) has said therefore seems to be definitely related to the placing of the antigen in an inflammatory area in which histiocytes and lymphocytes predominate

Following the discovery by French workers that the sensitizing property of killed mycobacteria is greatly enhanced in the presence of hydrocarbons Freund and McDermott (1942) imitated Dienes injection of protein into a tuberculous focus by blending horse serum and an emulsifier in paraffin oil containing killed tubercle bacilli and injecting the water in-oil emulsion Probably owing to the type of cellular response evoked by the liquid hydrocarbon and the mycobacterial substance an intense cutaneous reactivity to horse serum of delayed type resulted At the same time the output of circulating antibody became exaggerated and was sustained for many months

Therefore in animals so prepared a high degree of Arthus type reactivity is superimposed on the original Dienes effect Reference to either or both of these effects is variously intended when the term adjuvant effect of killed mycobacteria is used but it is necessary to distinguish between the effects due to the presence of mycobacteria and the effectiveness of antigen dispersed in hydrocarbon *without* the incorporation of mycobacteria or special mycobacterial wax So far as the concentration of circulating antibody is at issue the mycobacteria often can be omitted from the emulsion but the direc tive effect of the mycobacteria is needed in order to secure certain of the delayed type allergenic effects

When brain substance was incorporated in the Freund adjuvant it was found that a fatal demyelinating disease was induced in monkeys with disseminated lesions in brain and cord (Morgan 1946 Kabat Wolf and Bezer 1946) The effect was secured only

when mycobacteria were included in the emulsion The disease currently termed experimental allergic encephalomyelitis or EAE has been studied intensively in laboratory animals particularly in guinea pigs rabbits rats and certain strains of mice it has been viewed as a possible laboratory prototype of multiple sclerosis (see Kolb 1950) That the mechanism is independent of any subtle intraspecies antigenicity of brain substance has been nicely shown by Kabat *et al* (1949) who induced the condition in monkeys in response to a portion of their own brain substance removed in a prior operation The active material in brain resides chiefly in the white matter and has been isolated in several forms differing in their effectiveness in the various species of laboratory animals—proteolipid basic protein and most recently a dialyzable encephalitogen (Robertson *et al* 1962)

Delayed type reactions to brain are demonstrable by intradermal testing under appropriate conditions Thus Waksman and Morrison (1951) found dermal and corneal reactivity in tests with rabbit brain after homologous brain had been injected intradermally in Freund type adjuvant in order to prepare rabbits for EAE and Alvord and colleagues recently have found a skin reaction in guinea pigs to the encephalitogenic basic protein of homologous brain Such reactivity at 10 days had predictive value for early onset of EAE

The event of EAE can be deviated by injecting either brain or mycobacteria some days before administration of brain emulsified with mycobacteria apparently an initial production of antibrain antibodies effectively diverts the sensitization process as does prior sensitization to mycobacteria Thus Paterson and Harwin (1963) have found that injections of antibrain serum (19S antibodies) before sensitization can suppress development of EAE in rats Also the use of 6 mercaptopurine which suppresses immunologic activity will postpone the onset of EAE (Hoyer Good and Condie 1962)

Iso antibodies to brain substance are found irregularly one (19S) fixing complement with brain extracts and another type (with complement) being cytotoxic for cultured myelin and glial cells Serum does not transfer the condition to normal animals (cf Waksman and Morrison 1951) but transfer between rats and between isologous guinea pigs has been effected by means of lymph node cells (Paterson 1960 Paterson and Didakow 1961 Paterson and Bell 1962 Stone 1961) Rats developing EAE after cellular transfer also develop cytotoxic antibody but its relation to the lesions is not demonstrated However rabbits with induced EAE possess specific immunoglobulins that localize specifically in the myelin sheaths and glial cell membranes of cultured rat cerebellum causing marked demyelination within a few hours (Appel and Bornstein 1964)

An allergic neuritis has been shown to occur as another distinct entity when peripheral (sciatic) nerve is used instead of brain suggesting immunologic differences in myelins from the 2 sources (Waksman and Adams 1955 1956) Transferred recently to a tissue culture system of trigeminal ganglia the process of demyelinization has been observed to occur within 3 days when specifically competent lymph node cells were added to the culture and only infrequently when serum of the cell donors was used (Winkler 1964)

If testicular tissue instead of brain is incorporated in the emulsion another but non fatal iso allergic condition arises namely a marked degeneration of sperm cells from spermatogonia to the mature sperm this condition can be initiated by injection of material from one of the animal s own testicles removed beforehand (Voisin *et al* 1951 Freund *et al* 1953 1955) The active antigen is heat stable and contains polysaccharide or glycoprotein

With use of thyroid tissue likewise incorporated in mycobacterial adjuvant even auto allergic thyroiditis could be demonstrated by Witebsky and co workers rabbits being sensitized with portions of their own thyroid (cf Terplan *et al* 1960) Studied in a histocompatible strain of guinea pigs a chronic primarily lymphocytic disease was found to persist for 2 years after its peak severity at 2 months (Lerner and McMaster 1964) Delayed type reactions to thyroid were found at 5 to 7 days before onset of the disease and antithyroid antibody ap

rendered immune by inoculation with cowpox virus later responded to this or the closely related smallpox virus with a small local redness that appeared within 48 to 72 hours and then faded quickly This manner of response was interpreted as an allergy by von Pirquet and Schick in 1903 the role of hypersensitivity was convincingly demonstrated when Hooker (1929) injected heat inactivated virus into the skin of cowpox immunized individuals

The Frei test (originally an injection of diluted and heat sterilized pus from the lesion later of infected mouse brain) is now performed with antigen obtained from chick-embryo cultures A skin test betokening past infection with mumps virus has been developed by Enders *et al* (1945 1946) for the assessment of immunity Newer test preparations have been made of viral material grown in the allantoic fluid of embryonated eggs (Henle *et al* 1951)

Allergy in Parasitic Infestations In contrast with the above observations the form of allergic response that has received chief consideration in parasitic helminth infestations—for example schistosomiasis echinococcus disease filariasis trichiniasis and ascaris infestation—has been the immediate reactions of the wheal and erythema type found when the skin of the host is tested with extracts of the body substance of the same or related parasites usually antibodies can be demonstrated in the serum by serologic methods at the same time Asthma and rhinitis are sometimes elicited upon exposure to the specific agents While there is often an early reaction to hydatid fluid in echinococcus infestation it appears that the delayed type or *Casoni reaction* should be given chief diagnostic interpretation

In protozoan infestations skin tests have been less useful The only instance to be mentioned is leishmaniasis in which a tuberculin like reaction has been observed in skin tests made with an extract of cultured Leishmania

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The Adjuvant Effect of Tubercle Bacilli ' Auto-allergic Diseases

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as a flare in previously injected sites owing to a reaction with remaining traces of the sensitizing complex The full degree of sensitivity is best demonstrated by applying the same material (usually dissolved in a suitable vehicle) to a fresh area of the skin or by injecting intracutaneously either the incitant or an artificial conjugate antigen" made by joining the incitant to an appropriate soluble protein

The identification of sensitizing chemicals as those which can combine with proteins was demonstrated in the case of chloro and nitro-substituted benzenes (Landsteiner and Jacobs 1936) and conversely sensitive animals can be provoked to react by other chemically related compounds only when the latter can themselves combine with proteins (Eisen *et al* 1952) However not all classes of sensitizing substances exhibit so clear a basis for combination with proteins and alteration in vivo prior to combination must be assumed

Study in guinea pigs again points clearly to an altered behavior of lymphoid cells altered behavior of macrophages and a stimulus to form immunoglobulins and it emphasizes the cellular basis of contact sensitivity Upon transfer of living mononuclear cells the delayed type of dermal sensitivity not transferable with serum is imposed on normal guinea pigs exactly as in the case of tuberculin hypersensitivity (Landsteiner and Chase 1942 Haxthausen 1947 Nilzen 1952 Seebohm Tremaine and Jeter 1954) For this manner of sensitization Mitchison has introduced the term adoptive transfer The general histology of contact reactions after cellular transfer has been studied by deWeck and Brun (1956) With use of marker technics mentioned previously donor cells are found in reacting test sites of the recipient animal in small ratio to host mononuclear cells The chief question concerns the attraction of donated cells to test sites as to whether the ratio of donor to host cells differs in test sites from that in lymphoid tissue or the circulation Some workers have not found an elective concentration of labeled cells in cutaneous test sites greater than in the organs (Hamilton and Chase 1962 McCluskey *et al* 1963) but others have found specific accumulations (Najarian and Feldman 1961 1963 Kay and Rieke 1963) In respect to their findings Najarian and Feldman (1963) are inclined to view the ratios found as representing preferential *retention* of labeled competent cells in specific test sites rather than specific attraction

In man it has proved to be unexpectedly difficult to transfer contact type sensitivity with cells However experimental sensitivity to 2 4-dinitrochlorobenzene seems to have been so transferred into patients with hypogammaglobulinemia (Good *et al* 1957 1962) and sensitivity to dinitrochlorobenzene paranitrosodimethylaniline and perhaps poison ivy also (Epstein and Kligman 1957) If so the mode of sensitivity in man would be established In this connection one will recall the already mentioned experiment of Haxthausen in transplanting skin between identical twins one of whom had been sensitized with dinitrochlorobenzene When a satisfactory operational procedure is devised for this type of study in man one may expect positive results rather than negative or borderline instances of transfer that have been reported (Baer and Sulzberger 1952 Baer *et al* 1952 Harber and Baer 1961) Recent studies on exudates and blister fluids and cells adhering to cover slip windows at the site of specific reactions have shown a surprising richness in the types of white blood cells that are associated with passive transfer (Nexmand 1949 1950 Baer and Yanowitz 1952 Braunsteiner *et al* 1958 cf Fellner 1919)

In experimental sensitization with simple chemical allergens not only delayed-type hypersensitivity to contact with the chemical arises but immunoglobulins are synthesized as well (Chase 1947) these probably being chiefly of the 7Sγ_1 variety (see page 244) Allergic conversion precedes the appearance of antibody the interval shortening as sensitizing applications are increased in number For demonstration of the antibody usually by PCA the preparation of soluble haptene protein conjugates is required systemic anaphylactic shock often can be shown also

When cellular transfer is made from animals that possess delayed type hypersensitivity and are actively synthesizing circulating immunoglobulins the transferred cells can

peared slightly later By chemical modification of thyroglobulin that reduces the amount of circulating antibody, Miescher *et al* (1961) concluded likewise that delayed type hypersensitivity is the basis of the tissue damage rather than antibody

Auto immune nephrosis occurs when rats are sensitized by repeated injections of kidney extract with mycobacterial adjuvant and the disease can be transferred by means of lymph node cells (Hess Ashworth and Ziff 1962)

Contact Dermatitis*

The most common route of sensitization to natural products and defined chemical substances is the percutaneous one involving direct contact with the skin Existing irritations in the skin the kind of perspiration the chemical properties of the sensitizing material and the intensity of exposure are prime factors in sensitization In addition inherited factors influencing the degree of susceptibility such as are proved to occur in animals almost certainly exist in man Most frequently encountered by far is the delayed type of reaction with manifestations ranging from macules to frank epithelial injury and exfoliative dermatitis but reagins have been detected now and then and doubtless stimuli arise for synthesis of the more usual types of immunoglobulins as well

Inciting agents or contactants range from water soluble to fat soluble materials and can arise for example through the action of acidic perspiration on nickel-plated jewelry One widespread agent is the catechol derivative of the poison ivy plant urushiol which causes the allergy known as poison ivy dermatitis with frequent presentment of separation of the epithelial layer and formation of bullae Mild forms of contact dermatitis naturally can be mimicked by temporary superficial inflammations

The causative agents, not antigens as such appear to acquire antigenic capacity by combining with the tissues of the host (either directly or after some intermediary chemical alteration in vivo) as conjugated haptene protein structures analogous to the artificial conjugated antigens of Landsteiner Such haptene protein structures are believed to incite sensitization and to elicit subsequent reactions whenever the same or a chemically related substance presents itself by the proper route

Experimental investigation may be said to date from 1896 when Jadassohn first published his careful observations and introduced the patch test as a diagnostic procedure in the study of contact dermatitis The idea that such allergies represented individual and innate idiosyncrasies became untenable when Bloch and Steiner-Wourlisch (1926) pointed out that any individual could be rendered sensitive to the primrose plant (*Primula obconica*) if one simply used a concentrated extract of *Primula* leaves and a sufficient number of applications Some persons became sensitive after 1 application whereas others required several treatments and evidently the role played by heredity was reflected in this variable sensitivity The same workers next showed (1930) that guinea pigs as well could be sensitized to *Primula* and Mayer (1931) demonstrated that like human beings guinea pigs could be made sensitive to paraphenylenediamine a compound often used in the dyeing of furs The way was now open for animal experimentation for the manifestations of dermatitis in man and in sensitized guinea pigs were analogous even if not identical

Experimentation in guinea pigs has proceeded with use of 2 4 dinitrochlorobenzene picryl chloride *p* nitrosodimethylaniline *p* phenylenediamine 2 phenyl 4 ethoxymethylene 5-oxazolone substituted benzoyl and benzyl chlorides acid anhydrides Salvarsan urushiol and so on most being proved to be sensitizers of man also In both species sensitization is accomplished by repeated applications made on or into the skin and it may be necessary to provide a previously irritated cutaneous area for the treatments These technics sensitize the skin generally though in man there is some evidence for a slight delay before areas remote from the site of application acquire their sensitivity The *sensitivity arises usually in 5 to 10 days* and often is seen first on the 7th or the 8th day

* The reader is referred to Sulzberger (1940) Landsteiner (1945) Carr (1954) and Jadassohn (1932) for reviews which deal with historic and theoretic aspects

yet been evolved and except for spontaneous recession the sensitivity soon returns as is true in all other types of allergic manifestations Thus the degree of dinitrochlorobenzene sensitivity in animals could be lowered for only a short period by giving very large amounts of the allergen intravenously (Frey and Geleick 1962) For use in special studies it may be mentioned that guinea pigs can be *prevented* from undergoing specific sensitization to certain simple chemicals by certain elective experiences induced with the chemical before attempting experimental sensitization (intravenous injection of arsphenamine Sulzberger feeding of fat soluble allergenic chemicals Chase)

In man immediate type flare and wheal reactions to drugs have been found in several instances (Salvarsan formaldehyde phthalic anhydride chloramine T sulfathiazole and sulfadiazine) and often are accompanied by demonstrable reagins it is evident that these incitants which can give early type reactions would belong largely in the category of the more reactive compounds capable of rapid coupling to form haptene protein conjugates It may well be that as in the case of immunoglobulins in guinea pigs sensitized with chemicals these reagins may occur more commonly than has been recognized and that their detection by skin test may wait upon the use of suitable haptene-carrier conjugates For example using succinylated polylysine as a nonprotein (and nonantigenic) carrier of dinitrophenyl groupings Parker Kern and Eisen (1962) elicited flare and wheal reactions in a sensitive human subject as readily as with dinitrophenylated protein

In order to search for nonreaginic antibodies that may be present even in low concentrations in human beings exhibiting delayed hypersensitivity many novel methods have been proposed These deal chiefly with the use of allergen-coupled erythrocytes to detect antibody either by direct agglutination or by indirect tests for adsorption of immunoglobulin (cf Coombs Mynors and Weber 1950 Coombs and Fiset 1954) that would be similar in principle to the Middlebrook Dubos or the Boyden tests for the detection of antibodies in tuberculosis With certain very simple chemical allergens direct coupling to erythrocytes has been made (Johnson 1962 Bullock and Kantor 1964) these proving useful in animal experimentation With many allergens of man however it may not be possible to couple to protein without enslaving the immunologically determinant sites of the allergens

Delayed Sensitivity to Proteins

Seen only transiently in man and there termed the Jones Mote phenomenon delayed sensitivity to proteins has been studied extensively in laboratory animals After a single intradermal sensitizing injection of protein reinjection between the 3rd and the 5th days reveals a delayed type response initiated by small mononuclear cells but thereafter immunoglobulin appears and the delayed sensitivity is lost to view The time at which masking antibody will appear can be postponed by sensitizing with only small amounts of protein (3 to 30 mcg) or with protein precipitated by specific antibody (Uhr *et al* 1957) or with protein altered chemically by heavy coupling to haptenes In most instances the sensitizing procedure primes the immunologic apparatus silently so that a secondary response is evoked by the first subsequent skin test therefore these studies of delayed sensitivity are carried out usually with single tests made around the 8th to the 10th days The subject is reviewed in detail by Gell and Benacerraf (1961) and by Salvin (1962)

Examples of the experimental sensitization of man to proteins have been provided by the so called one shot treatment of allergic patients with allergens incorporated in mineral oil The inclusion in such emulsions of allergens to which the patient did *not* possess a specific hypersensitivity (wheal and flare type) has been found to induce long sustained delayed type reactions sometimes with ulceration (Feinberg *et al* 1960 Becker *et al* 1961)

Homograft Rejection

Skin and organ homografts placed in genetically unselected individuals are rejected regularly within 10 days to 3 weeks but not when donor and recipient are syngenic (identical twins highly inbred strains of mice and guinea pigs) The manner of

give rise to synthesis of antibody in the recipient as well as delayed hypersensitivity (Chase 1951) several days are required for detectable synthesis of antibody and the process stops at the time of the rejection of the donated cells The cellular transfers of delayed type hypersensitivity and of antibody synthesis are independent events indeed if donors have been stimulated with such antigens as bacterial extracts and diphtheric toxoid antibody synthesis can occur following cellular transfer (Harris and Harris 1951 Wager and Chase 1952)

The mechanism underlying drug sensitization appears to be involved intimately with the antibody producing system That in the establishment of delayed type sensitivities one is dealing with some part of the antibody-producing mechanism seems to follow also from some other observations First dermal sensitivity of the delayed type can be induced by an insoluble complex antigen (made of the chemical coupled to erythrocyte stromata) when it is used along with the Freund adjuvant (Landsteiner and Chase 1941 cf Chase 1954) Second when one feeds certain allergenic chemicals to non-sensitized animals there is induced a state of specifically depressed receptivity involving both the acquisition of delayed type *dermal sensitivity and the production of antibody* The animal acquires resistance to a subsequent dermal sensitization with this chemical and upon stimulation with conjugated homologous antigens bearing the same immunologically determinant structure there is pronounced depression in synthesis of corresponding circulating antibodies (see p 294) Again administration of methotrexate (aminomethylpteroylglutamic acid) which depresses the immunologic apparatus was found to block development of contact sensitivity and the primary antibody response (Friedman Buckler and Baron 1961)

Although sensitization as induced by percutaneous or intradermal application of chemical allergens shows specificity within the bounds set by their structures closer examination reveals that specificity is not related solely to the haptenic group that couples to self protein for adjacent portions of the carrier constitute part of the sensitizing structure (cf Benacerraf and Gell 1959 Gell and Benacerraf 1961a 1962b Salvin and Smith 1960 Benacerraf and Levine, 1962 Gell and Silverstein 1962 Leskowitz, 1963) In many such studies unfortunately sensitization has been effected with adjuvant incorporating highly coupled haptene protein conjugates, enough so to mask the role of the specific haptene moiety

In parallel with tuberculin hypersensitivity the macrophages of guinea pigs sensitized with simple chemical allergens display inhibition of migration in vitro when specific soluble haptene protein conjugates are added such as picrylated bovine gamma globulin in picryl chloride sensitive animals (Carpenter 1963 David *et al* 1964 Stinebring Axelrod and Trakatellis 1963) Again the role played by the carrier protein looms large

In studying the cellular changes occurring within auricular nodes chemical allergens being applied to the ear Turk and Stone (1963) found a marked increase of large pyroninophilic cells on the 3rd and the 4th days exactly at the time of incipient contact sensitivity Sensitivity ascended during the following 4 days as these large cells declined in number As postulated by Gowans (1962) the evidence was that the large pyroninophilic cells arose from small lym*phocytes and in turn gave rise to* immunologically competent small lymphocytes Cellular transfer of contact type sensitivity was regularly successful by the 5th day whole lymph node slices being used The relationship of the observed cellular changes to antibody synthesis on the one hand and on the other hand to development of cells effective in transfer of contact sensitivity is not known Processes known to interfere with the immunologic apparatus—specific tolerance cyclophosphamide—blocked the cellular changes although methotrexate permitted large cells to develop but still prevented sensitization

Finally it may be said that attempts at desensitization in contact dermatitis in man have met with limited success Some cases recede spontaneously a few others have been benefited by subcutaneous injection of the incitant in an oil vehicle (Caulfield 1936) or by cautious ingestion (Park 1944 Stevens 1945) A useful method has not

Converse and Tillett (1960) studied transfer factor (see p 277) prepared from lymphocytes of persons actively rejecting skin homografts (As mentioned previously such investigations are now possible only in man) The plan consisted in the transfer of acquired hypersensitivity transferred from an individual rejecting a particular skin to an individual newly grafted with the same skin and in the finding of accelerated rejection as compared with an indifferent skin graft Preparations made from the leukocytes of 3 persons who had rejected skin 4 times in succession caused specific accelerated rejection when injected into the intended recipients 8 days before or 3 days after grafting preparations from 3 individuals who had rejected a skin only twice were weaker and caused rejection only if infiltrated around the graft after vascularization on the 3rd day The factor in the lymphocytes was lost quickly and effective preparations could be secured only at the height of the rejection

As Stetson and Demopoulos (1958) showed circulating immunoglobulin is entirely adequate to ensure rejection of newly placed skin grafts but is of little effect on well established grafts serum complement seems to be required for the action of such antibody Other evidence supports this view as Gorer (1960) and Amos (1962) have emphasized the homograft reaction is complex and the basis of rejection may include in varying measure both delayed type hypersensitivity and humoral antibodies Demonstration that humoral antibody occurs in sufficient concentration to reject first set homografts has seldom been offered Homograft rejection was interpreted by Najarian and Feldman (1962 1963a b) to depend solely on an antibody mechanism labeled spleen and lymph node cells transferred from mice or guinea pigs that were stimulated by both skin graft and injection of spleen cells did not appear in the recipient s test graft although rejection occurred Finally a globulin-containing extract of the transfer cells was shown to effect rejection directly and in small amount Whether this is the sole mode of homograft rejection remains moot since antibody can be found in spleen cells of animals actively synthesizing it Yet Prendergast (1964) found that substantial numbers of labeled cells were present within all of 4 skin homografts during their rejection although the labeled cells arose only within one node that drained one of the homografts And indeed one finds a heavy infiltration of small lymphocytes in first set homografts

The participation of lymphocytes in both delayed type hypersensitivity and in events leading to antibody formation emphasizes the difficulty in separating the 2 functions Gowans McGregor Cowen and Ford (1962) and Gowans (1962) have shown impairment of primary antibody formation in rats by thoracic duct drainage and restoration by return of lymphocytes to the circulation a finding quite in accord with the role of lymphocytes in antibody production following stimulation with RNA from macrophages that had been incubated with antigen (Fishman and Adler 1963) Also Gowans *et al* studied transformation of rat small lymphocytes stimulated by placement in an alien host the small lymphocytes went to the spleen became large pyroninophilic cells and finally produced further small lymphocytes This sequence of changes occurs in the graft versus host reaction as well as in skin homograft rejection

The graft versus host phenomenon following cellular transfer implies that instead of expected homograft rejection transferred cells sometimes can respond to antigens of the recipient animal and affect the latter s well being (cf Simonsen 1962) This effect —termed homologous disease or runt disease or wasting disease—depends on the relative impact of antigenic stimulation and it varies considerably according to the choice of donor and the recipient strains e g among inbred mouse strains Homologous disease in the adult rat shows a great variety of allergic inflammations the reaction leading to dermatitis of host skin and rejection of autografts even while homografts are becoming established free of dermatitis (Stastny Stembridge and Ziff 1963)

In the area of allergic encephalomyelitis (EAE) rat lymphocytes taken on the 7th to the 11th days after the sensitizing injection of brain (in mycobacterial adjuvant) were found to cause destruction of glial elements of cultured puppy brain first ag

initial rejection and the speedier rejection of a later identical graft point to immunologic mechanisms The manner of rejection of a first set graft shows similarities with tuberculin type allergy as has been outlined by Lawrence (1957) and emphasized by experimental studies in animals (Medawar, 1958, Brent Brown, and Medawar 1959 Billingham Silvers and Wilson 1963) Second set grafts from the same donor are rejected more quickly Sooner or later attempts to regraft meet with failure to vascularize even briefly, as witnessed by the telltale white graft This final stage is attributed to interference by circulating antibody passive transfer of selected sera will duplicate the effect (Stetson and Demopoulos 1958) Sufficient antibody indeed can be directly damaging for first implanted cells (Siskind and Thomas, 1959 Gorer and Boyse 1959) These points of view—emphasis on delayed type hypersensitivity vs a role of immuno globulins—will be discussed in turn

Not only the lymphocyte but also the macrophage assumes a new role after the onset of sensitization The rejection of incompatible tumors by mice has emphasized the role of the macrophage phagocytosis being often prominent Monocytes transferred after such rejection can protect other mice from successful implantation even when washed free of iso antibody (Old *et al*, 1963) Another interesting example is the acute allogenic disease studied by Granger Weiser and Holmes (1963) when an ascites tumor indigenous to strain A mice is used to sensitize C57Bl mice the latters macrophages *not* lymphocytes are found to be capable of killing normal strain A mice within 40 hours by attacking primarily the capsule of the pancreas humoral antibody *not* being detectable

Consonant with the interpretation that delayed type hypersensitivity underlies first set rejections are experimental studies in animals showing similarities in the time of onset of the reaction the histology of the graft bed a failure to transfer with serum and especially a role of lymphoid cells in inciting rejection of tolerated grafts the cells being transferred from graft sensitized individuals (cf Billingham Silvers and Wilson 1963) Moreover such graft sensitized guinea pigs exhibit a delayed type sensitivity toward extracts of the donor s tissues and yield lymphoid cells that produce local hypersensitivity reactions when injected into the donor s skin (Brent Brown and Medawar 1962) Still further evidence for the role of delayed type hypersensitivity is offered in the rejection of skin homografts by fetal lambs approaching delivery although an ability to respond to antigens is developing slowly they reject skin regularly without the presence of discernible antibody (Schinkel and Ferguson 1953 Silverstein Prendergast and Kraner 1963)

Other evidence has been cited against a major role of humoral antibody in graft rejection When alien homologous cells are placed within a noncompatible host as target tissue but in chambers faced by special permeable (Millipore) membranes the cells survive (Algire *et al* 1954, 1957 Weaver *et al* 1955 Billingham and Silvers 1963, Billingham Silvers and Wilson 1963) However destruction ensues when immunologically competent cells are introduced into the chamber damage extending both to target cells and lymphoid cells (Weaver *et al* 1955) Many attempts to duplicate this result in vitro failed finally Rosenau and Moon (1961 1962 cf Rosenau 1963) used specifically competent lymphocytes in high ratio to target cells and indeed found cytolysis of mouse L-cell fibroblasts During the 1st day the slow attachment of perhaps 5 per cent of the lymphocytes was seen whereas lysis was most rapid between 36 and 48 hours Cytolysis of this type occurred without addition of serum complement In contrast humoral cytotoxic antibodies required a supplementary source of complement to effect lysis of such cells In similar exposures of cultured thyroid cells to competent lymphocytes Rose *et al* (1963) found thyroid cells to round up to retract and to cease growth yet the effect could be duplicated when lymphocytes of nonthyroid itis provenance were used in sufficiently great numbers Neither donors serum nor cell sap of the competent lymphocytes produced any effect on the cultured cells

Seeking in another way to demonstrate that homograft rejection depends on delayed type hypersensitivity Lawrence Rapaport

and Moon and only 5 to 35 per cent of lymphocytes in studies made by others were stimulated by tuberculin to synthesize gamma globulin in vitro (Pearmain *et al* and Hirschhorn *et al*) despite the competency of all the lymphocytes to form gamma globulin under the stimulus of bean leukoagglutinin (p 277) Yet it should be emphasized again that monocytes acquire new properties in consequence of sensitization as is shown well by in vitro inhibition of migration by exposure to the allergen and by the sequences seen in experimental listeriosis (p 278)

It seems curious that in man the transfer of microbial hypersensitivities is accomplished with so few cells as 0 15 ml moist volume and the hypersensitivity persists for so long (cf Lawrence 1960 Good Kelly Rotstein and Varco 1962) whereas in animals the hypersensitivity appears to be a function of survival of the transferred cells The easy separation of transfer factor from this small number of cells and the demonstration of dialyzability and small molecular weight should shortly increase our understanding of delayed type hypersensitivity in a way seemingly not possible by animal experiments

In an endeavor to rule out re excitation of existing but latent hypersensitivities—the constant hazard in dealing with microbial hypersensitivities—Rapaport Lawrence Millar Pappagianis and Smith (1960) studied transfer of coccidioidin hypersensitivity in man using as recipients persons far removed from an endemic area of coccidioidomycosis Transfer was successful reactions occurring to tests made 6 to 14 days later Control leukocytes from the single negative individual used gave only 1 moderate transfer among 9 recipients injected An extensive series of transfers with frozen human cells was made by Jensen Patnode Townsley and Cummings (1962) from persons variously sensitive to special tuberculins (PPD S PPD Battey and PPD Avian) recipients being tested on days 2 7 and 30 Transfers were multiple being positive in 70 per cent of 26 trials but positive only in 30 per cent toward PPD S In 6 instances sensitivity appeared to tuberculins that were not reactive in the donors and 1 negative control donor appeared to transfer reactivity to PPD B and PPD A Histoplasmin sensitivity proved to be difficult to transfer in this way even from good reactors

It has been noted that the special waxy substance of mycobacteria possesses a remarkable directive effect in inducing delayed type hypersensitivity to tuberculin and to other proteins or haptene protein complexes deliberately injected with it (cf White in Shaffer *et al* 1958) The directive effect may well be the attraction of cellular types such as monocytes and cells of the lymphatic series and the resultant formation of a local granulomatous response In addition the use of mycobacterial wax as adjuvant is accompanied by a widespread proliferation of plasma cell elements and an increased amount of antibody (White Coons and Connolly 1955) In the guinea pig indeed the effect of mycobacteria on antibody formation appears to be electively to induce synthesis of $7S_{\gamma}$ antibody (White *et al* 1963) Benacerraf *et al* 1963)

For the special case of chemical allergens Mayer (1957) has suggested that fibroid proteins such as collagen rather than soluble proteins may be the necessary carrier for the coupling that leads to delayed type hypersensitivity Mayer s evidence rests on his finding that procollagen may be substituted for mycobacteria or mycobacterial wax in a *special procedure of sensitizing the skin* by injecting a chemical allergen intraperitoneally and allowing coupling in situ The conclusion that the usual role of mycobacteria is to allow local accumulation of collagen as in the peritoneal cavity requires further examination

The hypothesis of cell bound antibody has been offered to explain the injury done by tuberculin to monocytes of the tuberculous host and by extension has been applied to lymphocytes which have been regarded as the prototypic cell in delayed type hypersensitivity The closest approach to cell bound antibody is the specific nonimmunoglobulin transfer factor of Pappenheimer and Lawrence However Bloom *et al* (1964) have concluded from metabolic alterations secured with Mitomycin C that transferred guinea pig cells must continue to metabolize in the new host in order to produce contact reactions Protein synthesis is suggested also

glutinating on to the glial elements and then largely disappearing within 4 days destruction of the cells occurred within 6 days The serum contained antibody that was absorbable by nonsensitized lymphocytes which then showed weaker contactual agglutination onto glial elements and apparently caused some destruction of the latter (Koprowski and Fernandes 1962)

The opposite of homograft rejection homograft tolerance, remains under active study because of its theoretic implications (cf Brent and Gowland 1963) The artifice of establishing tolerance through neonatal experience with cellular antigens (white cells of spleen peripheral blood lymph nodes) was established by the brilliant experiments of Medawar Billingham and Brent the subject has been reviewed in extenso by Brent (1958) and Hašek *et al* (1961) Tolerance can be established in certain cases in older animals by administering during the days of first contact with foreign tissue antimetabolites (p 294) that suppress the functioning of the immunologic apparatus Tolerance can be abolished by injecting isogenic cells either normal or with more prompt rejection those having a specifically acquired competence against the graft (Billingham Silvers and Wilson 1963)

Theoretical Considerations of *Delayed Type Hypersensitivity*

As we have seen the delayed type of hypersensitivity is associated in some way with alterations in cells lymphocytes and macrophages especially and with antigenic stimuli that lead in a measure to synthesis of circulating immunoglobulins Some unifying principles must underlie the delayed type of hypersensitivities that are apparent after microbial invasion after application of contactant chemicals after primary intradermal injections of soluble proteins and probably after the placing of homografts It has been suggested that delayed type hypersensitivity is the earliest and an immature stage in antibody formation directed against large surface areas of the inciting material in contrast with the production of mature antibody which recognizes limited areas or surface patches of antigen (Salvin and Smith 1960) Because it is found in appropriate instances that both forms of hypersensitivity can coexist 2 separable processes must occur in the immunologic apparatus For example, Gell and Hinde (1954) have suggested that delayed type hypersensitivity results from an antigenic stimulus that stops short of inducing plasma cell formation being confined to mononuclear cells Interestingly delayed type hypersensitivity occurs in many patients with agammaglobulinemia who have a pronounced deficiency in plasma cells and in the capacity to synthesize antibody Such patients show dermal reactivity following infections such as tuberculosis and Candida, or as a consequence of experimental sensitization by BCG vaccination or dinitrofluorobenzene or protein antigens coated with antibody (cf Porter, 1957 Good Bridges and Condie 1960) In all these cases cellular transfer with peripheral leukocytes was possible Yet some persons with agammaglobulinemia do not respond to allergization indeed one patient studied was found to be deficient in mononuclear cells during experimental inflammation An extensive review is presented by Good Kelly Rotstein and Varco (1962)

Emphasizing the role played by white cells in delayed allergies have been experiments showing that the allergic response is reduced significantly by depleting mononuclear cells through short term treatment with antilymph node serum or antimacrophage serum (Inderbitzen 1956 Wilhelm *et al* 1958, Waksman Arbouys and Arnason 1961 Pincus and Flick 1963), or through thoracic duct drainage prior to sensitization (Gowans *et al* 1962 Gowans 1962) or through thymectomy of rats at birth (Arnason Janković Waksman and Wennersten 1962) Affected are the allergic responses to tuberculin chemical contactants proteins inactivated vaccinia virus graft versus host reactions and in a measure allergic encephalomyelitis and homograft rejection

The number of lymphocytes stimulated by the process of sensitization is only a fractional part of the population as is evidenced by the finding of "competence" initially almost exclusively in nodes draining the site on which a sensitizing application is made Only 5 per cent of the lymphocytes attached to target tissue in the experiments of Rosenau

pig peritoneal cells Efforts are being made to relate this antibody (which appears to ab sorb to cell surfaces) to delayed type hyper sensitivity but no evidence has yet been presented of accomplishing passive transfer

THE SHWARTZMAN PHENOMENON

There remains to be mentioned a special sort of hemorrhagic necrotic reaction the Sanarelli Shwartzman phenomenon that can be produced in the skin and some other organs (chiefly the kidney) Accordingly the phenomenon is divided into the *local* and the *generalized* Shwartzman reactions It is not an antigen antibody mechanism but it pos sesses features in common with the Arthus reaction and its participation in acute dis ease processes is suspected

In this type of local tissue reactivity de veloped so ably by Shwartzman (cf review of 1937) sites in the skin of normal rabbits were prepared by injecting endotoxin-con taining culture filtrates of certain bacteria These sites would exhibit gross hemorrhage and necrosis within a few hours when cul ture filtrate was injected into the bloodstream between 8 and 32 hours later It was found that the skin preparatory factors and the reacting factors need not be identical or even immunologically related For example the local preparatory injection could be made with culture filtrates of *E coli* or *S typhosa* and the intravenous eliciting injec tion with meningococcal culture filtrates The active material in culture filtrates is identified with endotoxins of the bacteria the nitrogen free nontoxic lipopolysaccharide from *S marcescens* will both prepare and elicit the reaction The rabbit is the animal of choice for this experiment goats and horses show the effect guinea pigs respond irregularly and less intensely and mice and rats generally are found to be insusceptible However in certain specially bred strains of mice Shwartzman reactions have appeared upon an initial injection of bacterial polysac charide (Kelly *et al* 1957) In another highly inbred strain classic Shwartzman re actions were found to occur regularly (Arndt and Schneider 1960) As in the work of Kelly some of the mice reacted to the initial intradermal injection of endotoxin the authors relating this to preparation by gram negative microorganisms that were infecting the lung tissues without overt signs

It is possible to prepare the skin site with bacterial endotoxin or culture filtrates and to elicit the reaction by intravenous injection of certain nonbacterial materials—starch glyco gen agar or washed antigen antibody pre cipitates all of these being procedures which would affect guinea pig serum in vitro so that the serum would cause anaphylactoid shock Similarly endotoxin prepared skin will react locally when antigen antibody aggregates are allowed to form in vivo The reverse situa tion—namely substituting for the prepara tory injection a fully developed tuberculin reaction on a tuberculous animal and giving later an intravenous injection of a potent Shwartzman filtrate or even tuberculin—also results in a typical Shwartzman reaction (Freund 1934 guinea pig Stetson 1955 rabbits) tumor tissue in mice is said to offer a naturally prepared situs for Shwartzman effects since it responds with inflammation when potent bacterial filtrates are injected intravenously

From studies chiefly by Thomas and Stet son (1949) and Stetson (1951a b) it is known that after the preparatory injection polymorphonuclear leukocytes accumulate locally in the tissue around the smaller veins as cuffs and probably owing chiefly to the metabolic character of exudate poly morphonuclear leukocytes abnormal quan tities of lactic acid are produced there through aerobic glycolysis Changes (histologically inapparent) then occur in the adjacent vascular endothelium perhaps of a sort that render this tissue sensitive to the action of tissue protease Then upon the intravenous eliciting injection a peripheral vasoconstric tion occurs impairing the blood supply white cells and blood platelets clump and be come sequestered especially in the lung and attach markedly to the damaged endo thelium In the latter areas the aggregates form leukocyte platelet thrombi and actually occlude the small veins and the capillaries The death and the disintegration of involved vessels and of cells within the following 2 or 3 hours follow in the pattern of developing

by Janković and Dvorak (1962) in their finding that ribonuclease will inhibit temporarily interaction between competent rabbit lymph node cells and target cells

The relation of delayed type hypersensitivity to antibody-synthesis remains a major question even if the interpretation of 2 separate processes is correct Pappenheimer (cf Shaffer *et al* 1958) proposed that delayed type hypersensitivity may occur always as a first step in the production of antibody, representing a mechanism analogous to induced enzyme formation, that prepares the altered cells to produce circulating antibody electively upon a subsequent experience with the same antigen Yet delayed type hypersensitivity is not always found to precede antibody formation and the conclusion that this hypersensitivity becomes superseded when circulating antibody appears would require continuous and silent stimulation of newly maturing cells to sustain delayed type hypersensitivity

In seeking explanations for delayed type hypersensitivity in terms of antibody Burnet once suggested that eventually there is produced and put into the bloodstream a special antibody of such variety that it binds to the tissues with extreme readiness and is seldom to be encountered in the blood in an amount sufficient to permit passive transfer A search for such antibody was made by Cole and Favour (1955) and by Rauch and Favour (1960) in the plasma of tuberculin sensitive guinea pigs the equivalent of 80 ml being transferred At times a positive nontypical reaction to tuberculin was seen in the recipient but this approach appears to have been abandoned

Also in line with Burnet s suggestion is a hypothesis of delayed type hypersensitivity offered recently by Karush and Eisen (1962) and intended to unify all varieties of delayed hypersensitivity—tuberculin reactions contact dermatitis homograft rejection delayed type ocular reactions inhibition of cellular migration and other manifestations of tissue damage occurring in vitro (The hypothesis does not concern itself with the transfer factor of Lawrence and Pappenheimer) According to this concept the delay in the reaction appearing at a test site on a sensitive individual represents the time for synthesis of a very highly avid antibody its rate of production being sufficient to bind 10 per cent per hour of the antigenic complex in the test site during a 24 hour period, thereby producing gradually ascending inflammation The idea calls for stimulation and continued production of antibody possessing an exceedingly high avidity but at a concentration not to exceed 1×10^{-10} molarity in the serum (consequently not allowing transfer by serum) The test injection is pictured as immediately producing small amounts of soluble antigen antibody complexes These taken up from the circulation by the spleen and the nodal tissues would stimulate a recall synthesis leading to perhaps 4 fold as much newly synthesized antibody within 24 hours as had been present in a sensitized guinea pig before testing the rate of synthesis would necessitate a much more rapid turnover of the antibody than of total gamma globulin After reaction the production of this highly avid antibody would drop back to the previous undetectable concentration Adoptive transfer of contact type hypersensitivity would be ascribed to synthesis by the transferred cells of high affinity antibody Similarly cytotoxicity in tissue culture would result from antibody synthesis by the competent cells

As corollaries it would follow that (1) the highly avid antibody must appear in 5 or 6 days after injection of chemicals like dinitrochlorobenzene when the sensitizing injection was intradermal and not appear when the injection was intramuscular (2) the directive effect of mycobacterial adjuvant with haptene protein complexes would be explainable as causing production of highly avid antibody and (3) the rate of its synthesis must drop as soon as an animal has become sensitive although the synthesis of less avid antibodies is not hindered Whatever the merits of the hypothesis may be it will stimulate a more intensive study of antibody patterns

Another type of antibody cytophilic antibody has been described (cf Boyden 1963) being found in very low concentration in serum, the antibody is heat stable and absorbs to cell surfaces such as guinea

haps be described as the Frei Sulzberger Chase phenomenon The subject is of particular interest because certain noncutaneous routes of administering sensitizing compounds will alter the status even of adult animals deviating later attempts to induce delayed type hypersensitivity towards the same compound (reviewed by Chase 1959 1963 cf Coe and Salvin 1963 Battisto and Chase 1963) This type of unresponsiveness persists for 10 or more months and it is accompanied by *depression* of antibody synthesis The mechanism is unknown

ANTIHISTAMINES

Emphasis on the role of histamine in anaphylactic shock and in allergic reactions of immediate type excited the hope that the physiologic consequences of antigen antibody interaction might be avoided if the liberated histamine could be rendered ineffective Various approaches have been explored such as administration of the enzyme histaminase immunization by an artificial antigen containing histamine coupled to a protein and administration of special compounds possessing antihistaminic properties This last approach has provided a powerful adjunct in the treatment of allergic conditions The compounds influence in some degree other physiologically active substances (Loew 1950 Feinberg *et al* 1950) They are presumed to function as competitive inhibitors for the sites affected by histamine and they can be effective only as long as they can be maintained in tissues at an adequate level with respect to the histamine concentration

As with other proprietary compounds the products possess not only their trade names but also type names thus Pyribenzamine is one brand of tripelennamine hydrochloride Histadyl and Thenylene are different brands of methapyrilene hydrochloride Benadryl is a brand of diphenhydramine hydrochloride and so on (Cf Wilhelm 1961 for discussion of chemical classes)

The *evidence* for the protective action of these compounds against the effects produced in a normal animal by administration of histamine is impressive it extends to protection of the isolated guinea pig gut or uterine horn from contraction by histamine and to inhibition of vascular effects of histamine in the rabbit and other animals to the protection of guinea pigs from multiple lethal doses of histamine and from the bronchospasm caused by histamine aerosol At the same time it is found that most of these compounds do not modify the gastric secretogogue action of histamine When the antihistaminic compounds are tested for ability to protect anaphylactically sensitized guinea pigs against systemic shock by an intravenous injection of antigen larger amount are necessary (3 mg /Kg down to 1 mg /Kg) than are needed to deviate the effects of purely histamine shock

The variety in formulae has been welcomed because of individual reactions and requirements (cf Wilhelm 1961) All these compounds can produce undesirable side reactions such as drowsiness dizziness disorientation and gastrointestinal disturbances and occasionally (with overdose) even death hence the use of these substances by allergic individuals should be under medical supervision Often the antihistamines prove to be valuable in cases of allergic urticaria and allergic rhinitis although asthma especially is apt to be resistant Since the compounds function also as local anesthetic somewhat more so in this respect than procaine they may relieve the itching in poison ivy dermatitis but they do not modify in fundamental manner reactions that are of the delayed type

ACTH AND CORTISONES

Following the introduction of porcine ACTH (pituitary adrenocorticotropic hormone) in 1946 many diverse disease processes were found to be markedly ameliorated and among these were nearly all varieties of allergic manifestations Shortly afterward compound E of the adrenal cortex (cortisone) became available and was found to influence markedly the allergic state Other compounds including partially synthesized materials have since been developed (cf Jailer 1955) These materials include hydrocortisone 5 fluorohydrocortisone prednisone prednisolone methyl prednisolone dexamethasone and betamethasone They prove to be effective in relieving intrinsic and extrinsic asthma and as salves they control allergic eczema as well Treatment with

Arthus reactions The characteristic and prominent hemorrhage in the prepared site would follow as a consequence of necrosis of the vessel walls

As Rall *et al* (1957) point out, adrenergic vasoconstriction accompanying the eliciting injection could enhance local damage since epinephrine is known to exaggerate Shwartzman effects After making parallel studies on tuberculin sensitive rabbits injected with Old Tuberculin Stetson (1955) has suggested that the biologic activity of endotoxins may reflect a delayed type natural hypersensitivity of the rabbit to gram negative bacteria Evidence has been found also for this conclusion in rabbits hypersensitive to staphylococci (Atkins 1962b) Perhaps one could say that the Shwartzman event is to the Arthus and the delayed type allergies what the anaphylactoid is to the anaphylactic shock namely parallel and interwoven mechanisms for obtaining the same ultimate tissue response

Besides this *dermal* Shwartzman reaction the second aspect is the *generalized* Shwartzman reaction in which both injections must be given intravenously characteristically from 6 to 8 hours after the eliciting injection there is deposition of a fibrinoidlike homogeneous material particularly within the glomerular capillaries of the kidney which leads to occlusion of the circulation and results in bilateral renal cortical necrosis The determining event in initiating the generalized Shwartzman phenomenon appears to be intravascular coagulation The phenomenon can be duplicated when thrombin is injected directly into the renal arterial circulation (Robbins and Collins 1961) thus circumventing the normal action of the reticuloendothelial system (RES) in clearing fibrin (Lee 1962) Neither the local nor the generalized Shwartzman phenomena occurs if leukopenia has been induced previously as by nitrogen mustard

There has been active speculation that natural infections with bacteria may cause tissue preparation and that Shwartzman effects can occur owing to reacting factors provided by the infection itself by a proximal antigen antibody reaction or by a variety of other nonspecific stimuli this sequence has been surmised to be one possible factor operating in bacterial allergy and in the genesis of conditions such as pulmonary abscess or hemorrhagic lesions seen in meningococcemia (Black-Shaffer *et al*, 1947) Recently it has been found that when *pregnant* rats are placed on a diet of oxidized cod liver oil the pathology of the generalized Shwartzman phenomenon is duplicated without use of endotoxin (Stamler, 1959 McKay and Wong 1962)

MODIFICATIONS IN THE ALLERGIC STATE

Certain pharmacologically active materials serve temporarily to mitigate or to suppress allergic episodes of man Included are the so called antihistamines pituitary adrenocorticotrophic hormone (ACTH) cortisone (compound E), hydrocortisone, fluorohydrocortisone prednisone prednisolone and still others

Also for experimental purposes one can hold the immunologic apparatus in check for some days by means of antimetabolites such as 6 mercaptopurine methotrexate cyclophosphamide and the like administered just prior to and for some days after institution of a sensitizing procedure (cf Schwartz 1963 Hoyer Good and Condie 1963) Notable effects have been seen also by applying sublethal x irradiation for its effective depletion of lymphoid cells or by lowering the population of monocytic cells before intended sensitization by giving antilymphocyte or antimonocyte sera or by subjecting animals at birth to thymectomy thereby depressing the full development of lymphoid tissue

Under special conditions the immunologic apparatus can be rendered unresponsive to stimulation by particular antigens and yet retain responsiveness toward others (reviewed by Chase 1959 cf Bussard consulting editor 1963) Both synthesis of immunoglobulin and manifestations of delayed type hypersensitivity can be influenced

Of the many facets of immunologic tolerance induced tolerance to homografts already has been considered and mention need be made here only of induced unresponsiveness to sensitization with simple chemical compounds in guinea pigs which can per

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ACTH has been shown to mitigate dyspnea and bronchoconstriction in man toward experimentally administered histamine and Mecholyl (Rose 1950) Likewise, these compounds are of benefit in the collagen vascular diseases thought to be allergic in nature

Cortisone and hydrocortisone were investigated at once and were found to possess as Germuth (1956) has remarked a marked antiflammatory effect owing perhaps to a vasoconstrictive action that leads to reduction in blood flow and inhibition of capillary permeability there is increased vascular tone lessened diapedesis and inhibition of lymphocytic reproduction These compounds do not affect the interaction of antigen and antibody for passive Arthus reactions can be evoked typically (Germuth and Ottinger 1950) although synthesis of antibody appears largely to cease upon hormonal treatment (cf Germuth *et al* 1951) reduction in circulating antibody does not run parallel with clinical improvement

Actively sensitized dogs and mice exhibit diminution in anaphylactic shock In rabbits both the Shwartzman reaction and serum disease are inhibited Intensive treatment with the hormones also diminishes delayed type reactions as to tuberculin A prototype has been seen in the markedly lessened inflammatory response of intensively treated animals injected with oil of turpentine (Osgood and Favour 1951 cf Gell and Hinde 1951)

Stringent medical supervision is required with these highly useful agents particularly with regard to occurrence of peptic ulcer diabetes and osteoporosis The physician must weigh carefully the temporary benefits that the patient may derive against the grave disadvantages that come from exhaustion of the adrenals and impairment of the normal hypothalamus pituitary adrenal axis Since inflammatory responses are suppressed there is the hazard with continued use that serious bacterial and fungal infections may become established With ACTH there is occasional allergization which usually is specific for the species of origin A hormone fastness also has been encountered infrequently

Although initial symptomatology usually returns with cessation of treatment in instances in which the offending allergen can be expected to disappear within a definitely limited time from the environment (pollens) or from tissue sites (e g following a reaction to injected penicillin), there may be no further symptoms after treatment is terminated

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12

Endotoxins

The injection of cell walls of gram negative bacteria into experimental animals is followed by an array of toxic effects These include fever leukopenia skin reactions the Shwartzman phenomenon shock and death depending on the dose of material and the the route of its administration The active material or endotoxin can be extracted readily from intact bacteria or from purified cell wall preparations Endotoxin preparations derived from one bacterial species may differ quantitatively in potency from preparations from other bacteria but the effects produced by all endotoxins are qualitatively the same The lethal dose of endotoxin for most laboratory animals is of the order of 1 mg /Kg of body weight while the intravenous injection of less than a microgram causes a brisk fever in man rabbit and other species Because of the possibility that bacterial endotoxins are involved in various human disease states and because of the variety of the effects which they produce (Thomas 1954) much investigation has been carried out on the chemical nature of endotoxins and on the mechanisms by which they act

Extraction of gram negative bacterial cell walls with trichloracetic acid or aqueous phenol yields a toxic macromolecular complex of protein lipid and polysaccharide with a molecular weight of the order of 1 to 10 million Protein can be eliminated from such preparations without apparent loss of toxicity and much of the lipid can be removed from the resultant lipopolysaccharide without significant loss in endotoxin activity (Ribi *et al*, 1964), but no truly lipid free preparations have shown endotoxin activity

The polysaccharide of the endotoxin is the somatic or O antigen of the bacteria Purified preparations of these polysaccharides retain their antigenicity, and analysis of the products of hydrolysis of these has provided a basis for the chemical interpretation of their immunologic specificities In general the polysaccharides of all endotoxins contain D glucosamine heptose and glucose most also contain one or more additional sugars such as pentoses desoxyhexoses dideoxyhexoses mannose or galactosamine The patterns in which these sugars occur have permitted the arrangement of *Salmonella* into a system of chemotypes (Kauffmann *et al* 1960) which corresponds nicely to the older serotypic classification

It has been suggested that the toxicity of the lipopolysaccharide endotoxin resides primarily in the lipid and that the polysaccharide moiety merely serves a carrier function (Westphal and Lüderitz 1954) but little evidence has been forthcoming to support this view Indeed there is some doubt that endotoxins possess any intrinsic pharmacologic activity or toxicity as do exotoxins It has been pointed out that in many ways the effects produced by the injection of bacterial endotoxins into normal animals resemble the effects produced by the

injection of other antigens into specifically hypersensitive animals (Stetson 1955) For example the biphasic febrile response of normal rabbits to endotoxin is quantitatively and qualitatively much like that of tuberculin sensitive rabbits to injections of tuberculoprotein The skin reaction of normal rabbits to the intradermal injection of endotoxin closely resembles in its timing and histopathologic features the classic combined allergic reaction of Gell and Hinde (1954) The transient leukopenia thrombocytopenia and vasomotor phenomena seen during systematic reactions to endotoxin have their counterpart in typical anaphylaxis and Weil and Spink (1957) have pointed out many similarities between anaphylactic shock and shock caused by endotoxin

The Shwartzman phenomena (Shwartzman 1937) once were considered to be manifestations of a unique toxic property of endotoxins In the local Shwartzman phenomenon an intradermal injection of endotoxin is followed in several hours by an intravenous injection resulting in severe hemorrhagic necrosis at the site of the intradermal injection The generalized Shwartzman phenomenon is produced by 2 intravenous injections of endotoxin separated by a suitable time interval and results in the production of massive bilateral renal cortical necrosis It has been possible to reproduce both of these phenomena with other antigens in suitably sensitized or immunized animals (Lee 1963)

Therefore it may be that endotoxins possess biologic activity only by virtue of their antigenicity Obviously relevant to this problem is the demonstration by Schaedler and Dubos (1961 1964) that the reactivity of mice to endotoxins is very much dependent on or conditioned by the bacterial flora of their intestinal tracts Mice reared in an environment free of the usual enteric pathogens lack normal susceptibility to endotoxin On exposure of such mice to gram negative bacteria, they quite promptly become susceptible to the lethal effect of endotoxin These observations may indicate that susceptibility to endotoxin is due to sensitization by somatic antigens of intestinal bacteria The natural antibodies to somatic antigens of various enterobacteria probably arise as a consequence of active antigenic stimulation of the host by intestinal bacterial antigens as newborn animals or animals reared under germ free conditions have markedly reduced levels of such natural antibodies A number of considerations suggest that these antibodies may be the mediators of the various biologic activities of endotoxin (Landy and Weidanz 1964, Lee and Stetson 1960)

The repeated injection of sublethal amounts of an endotoxin results in a remarkable state of tolerance in which further injections of the same or any other endotoxin fail to produce their usual effects While this phenomenon has some operational and other similarities to the phenomenon of allergic desensitization the parallelism is not exact Endotoxin tolerance appears to depend in large part on the development of a marked increase in the phagocytic capacity of the fixed cells of the reticuloendothelial system with a correspondingly rapid clearance of endotoxin from the circulation Blockade of the reticuloendothelial system by Thorotrast or other colloidal material promptly restores the susceptibility to endotoxin (Beeson 1947) However tolerance also seems to depend to an appreciable extent on the prompt and vigorous humoral antibody response occasioned by the repeated exposures to the highly antigenic endotoxin Serum of tolerant animals is capable of conferring partial tolerance on normal recipients (Freedman 1960) Thus the more rapid removal of endotoxin from the circulation of a tolerant animal is probably due to prompt interaction with antibody with subsequent phagocytosis of the antigen antibody complexes The relative importance of the humoral and the cellular factors is still a matter of controversy but there is now no doubt that a tolerant state develops in man during typhoid fever (Greisman *et al* 1961) and the mechanism of endotoxin tolerance has become of more than academic interest

This brief account has made no mention of the capacity of bacterial endotoxin to elicit tumor hemorrhage to enhance the antibody response to unrelated antigens to initiate intravascular coagulation and to induce severe functional impairment of the

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12

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13

General Host Responses to Infection

Our purpose in this chapter is to describe and characterize certain physiologic reactions produced by infection in the mammalian host, particularly in man These host responses are fever and other alterations in control of body temperature circulatory changes hematologic abnormalities subjective feelings such as weakness and malaise and a miscellany of hormonal and other metabolic reactions The immune response and the actions of bacterial exotoxins are reasonably classified as systemic reactions to infection but are more specific than these others and are dealt with in detail elsewhere in this book

In a sense these systemic responses to infection can be regarded as the actual substance of disease They underlie the sensations which the individual human patient recognizes collectively as illness and they are the basis for the manifestations recognized by the clinician as the signs and symptoms of disease

The various local events initiated by implantation survival and replication of pathogenic microorganisms in the tissues of a susceptible host have been detailed in preceding chapters Inflammation and attendant phenomena such as phagocytosis are in*evitable biochemical and biophysical result*ants of the interaction of parasite and host However it is most difficult to avoid a teleologic or perhaps pragmatic concept of them as mechanisms of host resistance as defenses The local defense is often so efficient that no injury is recognized or that the only stigma is a subsequent immunologic response In other words the infectious process remains *subclinical* At other times local inflammation becomes grossly evident and signals disease by erythema pain swelling and impairment of function Finally in a minority of instances when local defenses are insufficient to contain the parasite or its products the general responses of the host come into play clinical illness is recognized and *infection* has evolved into *systemic disease*

The local inflammatory response s nonspecific in the sense that its general pattern is the same for injury produced by microbes antigens to which the host is allergic chemical toxins or physical trauma Similarly any or all of the systemic reactions of the infected host can be elicited by noninfectious disorders ranging from thrombosis to neoplasia The ease with which local exudation and phagocytosis are interpreted as host defenses contrasts with the difficulty experienced in demonstrating that many of the general responses described below have a role in protecting the host

FEVER

In man and in many animals the febrile response is a regular accompaniment of systemic disorders of many etiologies involving pathologic processes of almost every type So frequent is fever and so easily is it detected

reticuloendothelial system A comprehensive source of more detailed information concerning bacterial endotoxins has been provided recently by Braun and Landy (1964)

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importance of humoral mechanisms in the resistance

The importance of avoiding endotoxin contamination in the experimental study of fever and the additional usefulness of endotoxin fever as a standard of comparison in the laboratory will be evident in the discussion to follow

Finally the fact that endotoxin is injurious to tissue must be borne in mind The delay between injection of endotoxin and the onset of fever led Beeson to suggest some years ago that the pyrogenic action of endotoxin might be secondary to tissue injury and that the lag period might be the time required for release of endogenous fever producing material from injured cells

Endogenous Pyrogens The extensive work of Menkin on inflammation included the isolation from sterile exudates of a substance *pyrexin* capable of producing high fever in animals Menkin postulated that pyrexin was the product of tissue injury responsible for the fever that accompanies many inflammatory states Comparison by Bennett and Beeson of the properties of pyrexin and of ordinary bacterial endotoxins indicated that their activities were identical in every respect and made it probable that that pyrexin owed its potency to endotoxin contamination The properties of pyrexin are unlike those of any of the endogenous pyrogenic substances that have been isolated in recent years

Proceeding on the assumption that normal tissues might contain a pyrogenic material which is released at the time of injury Bennett and Beeson attempted to extract fever producing substances from the tissues of the normal rabbit Of all cells and tissues tested only the polymorphonuclear leukocyte was found to contain a pyrogenic substance Extracts of polymorphonuclear leukocytes produced a brief monophasic fever after a very short lag period and animals continued to respond to daily injections of this material with the same amount of fever Furthermore leukocyte pyrogen was fully active in animals made tolerant to bacterial endotoxin and was destroyed by mild heating

Atkins and Wood searched for endogenous pyrogenic substances in animals given endotoxin Immediately after injection circulating blood was found to contain a pyrogen which resembled in every respect the endotoxin originally injected The blood then became nonpyrogenic but somewhat later another pyrogenic material appeared in the blood usually reaching a peak 2 hours after injection This second pyrogen usually referred to now as endogenous serum pyrogen was found to possess properties entirely different from those of the endotoxin originally injected Indeed by every biologic test it was identical in activity with leukocytic pyrogen Since one of the initial responses of an animal to injection of bacterial endotoxin is the development of a profound neutropenia it was suggested that endotoxin produced its febrile response by initial injury to leukocytes and possibly to other tissues with release of an endogenous tissue pyrogen which had a direct action on the cerebral temperature regulating centers By perfusing the carotid artery King and Wood were able to confirm a direct action of endogenous pyrogen on the brain

Additional studies indicate that the febrile responses to pneumococcal infection to hemolytic streptococcal infection to injection of myxoviruses to injection of antigen into hypersensitive animals and to injection of certain strains of staphylococci are mediated by an endogenous pyrogenic material which appears in the serum of the febrile animal and remains there throughout the febrile period While an initial neutropenia is not as striking in all of these experimental fevers as it is with endotoxin there is ample reason to believe that an important source of the serum pyrogen is the polymorphonuclear leukocyte

Recently Atkins and Snell have demonstrated thermogenic substances in normal rabbit tissues including skeletal muscle liver and kidney The pyrogens are demonstrable only after extraction of relatively large amounts of tissue and the febrile response does not completely imitate that obtained with leukocyte extract However the fact that pyrogen may be derived from cells other than the polymorphonuclear leukocyte is now established and many of the fevers in disorders that appear to involve no direct injury to leukocytes eventually may be explainable on this basis

that it is one of the first signs looked for when illness is suspected Practically speaking fever can be regarded as one of the dividing lines between health and disease

Pyrogenic Agents

Some bacteria fungi and viruses produce high fever in animals or man However other microorganisms are lacking in any immediate pyrogenic activity, furthermore fever is a common accompaniment of diseases that apparently do not involve invasion of the body by a biologic agent The obvious common denominator in disease is injury to tissues and cells Consequently it has long been believed that fever is the result of release of endogenous pyrogenic substances from injured tissues Evidence accumulated in recent years supports the existence of this mechanism particularly in fevers accompanying infectious diseases but detailed knowledge of the nature of the derangement of temperature regulation in many disorders is incomplete

Bacterial Pyrogens Until the 3rd decade of this century there had been many reports *that seemed to support unequivocally the* hypothesis that the products of injured tissue were responsible for the fever of disease However the discovery by Florence Seibert of bacterial pyrogens cast doubt on the significance of all earlier demonstrations of thermogenic activity in biologic materials and furthermore made it feasible to design valid experiments on the pathogenesis of fever It had been known for a long time that the intravenous infusion of a wide variety of fluids such as saline or glucose was likely to be followed by a shaking chill and several hours of fever Bacterial etiology of these fevers had been ruled out since sterile fluids retained pyrogenicity the tendency was to attribute injection fevers to an intrinsic property of the solution in use Seibert and her colleagues demonstrated that pyrogenic fluids contained small amounts of bacterial products and that the pyrogenic activity of these materials was unaffected by ordinary means of sterilization, including autoclaving These materials formerly referred to as bacterial pyrogens are now known to be the endotoxins of gram negative organisms Modern methods for the preparation of fluids for intravenous use are dependent on the avoidance of contamination because of the extreme difficulties encountered in removing these substances once they have made their appearance Bacterial endotoxins have been purified and are known to be lipopolysaccharides *Not only are they capable of inducing high* fever when injected into man or animals but also they possess other biologic activities including the induction of severe leukopenia hemorrhagic necrosis (Shwartzman phenomenon) shock and even death (Chap 12)

The ubiquity, the heat stability and the potency of these thermogenic bacterial products made it evident that the search for endogenous pyrogens released from injured tissue must employ technics that would avoid endotoxins It became mandatory for investigators to demonstrate that any so-called product of tissue injury with pyrogenic properties was not simply contaminated with exogenous bacterial substances

Endotoxin Fever The febrile response of most laboratory animals to endotoxin is rather characteristic There is a delay between injection of the material directly into *the circulation* and *onset of fever* *In man,* this lag period may be as long as 90 minutes but in most animals it is somewhat shorter With daily injections of the same dose of endotoxin there develops a state of resistance to its pyrogenic effect and this *tolerance* persists as long as regular injections are continued When injections cease the ability to respond with high fever is restored quickly The injection of particulate materials such as certain dyes suspensions of carbon or colloidal thorium dioxide (Thorotrast) increases the susceptibility of the normal or tolerant animal to the effects of endotoxin Endotoxin tolerance is not dependent on the classic antibody response and is nonspecific in the sense that an animal made resistant to an endotoxin from one bacterial species will also exhibit tolerance to endotoxins from nonrelated organisms Tolerant animals clear injected endotoxin from the bloodstream more rapidly than do normal animals the clearance involves the reticuloendothelial cells of the liver and the spleen However tolerance can be passively transferred by serum (the transfer is only partial but is definite) indicating the possible

mals with chronic pyelonephritis and in human subjects with chronic urinary tract infection produced by gram negative bacilli

The injection of endotoxin into the circulation or its release from organisms multiplying in tissue is almost certainly followed by injury to polymorphonuclear leukocytes and possibly to other cells with the release of pyrogenic material The endogenous pyrogen or pyrogens then apparently act directly on the cerebral centers governing body temperature and fever follows If the number of circulating granulocytes is reduced by administration of nitrogen mustard the amount of serum pyrogen demonstrable after infection of endotoxin is reduced materially (see Gillman Bornstein and Wood for summary of all studies) However such results are difficult to interpret in view of the debilitating effect of nitrogen mustard and the reduction in febrile response is perhaps not specific

Injection of endotoxin into the lateral ventricle of dogs into the *cisterna magna* of dogs or into the subarachnoid space of rabbits elicits high fevers which come on after little or no lag period which are unaccompanied by peripheral leukopenia and to which animals develop no tolerance despite repeated injections (Bennett Petersdorf and Keene) While further work is needed on this subject it seems likely that the action of endotoxin in producing fever may involve more than one mechanism and that endotoxin may be able to act directly on the cerebral centers

The Significance of Fever in Infectious Disease

Fever is no more than a physical sign of disease and therefore can be interpreted only in light of all information available to a physician However close attention to the type of fever and to certain accompaniments of fever can yield valuable diagnostic information The fever in many infectious diseases is so irregular that it offers little help in diagnosis but in some instances it can serve as a reliable guide to the physician in planning diagnostic procedures in evaluating therapeutic response and in indicating relapse or recurrence

Important points to be noticed in evaluating fever as a physical sign are height diurnal pattern type of temperature curve association with bradycardia association with chills and the occurrence of certain complications

Height of Fever Very high fevers may be seen in patients with meningitis poliomyelitis viral encephalitis and of course in other disorders of the central nervous system The hyperthermia observed under these conditions probably is a result of some direct involvement of the hypothalamus by the disease process

When the oral temperature in an adult with an infectious disease exceeds 105 F one should suspect the possibility of tuberculosis malaria relapsing fever or a gram negative bacillary infection such as tularemia brucellosis typhoid fever or pyelonephritis

There are some infections in which fever is not as prominent as might be expected For example the febrile response in diphtheria is usually less than 102 F and in the presence of pharyngitis with high fever streptococcal or viral infection is more likely Certain types of cutaneous ulceration such as anthrax and sporotrichosis are characterized by low grade fever and this may help the physician to differentiate these from tularemia or glanders Among mycotic diseases cryptococcosis is characteristically an afebrile disease

Type of Temperature Curve Elevation of body temperature by disease usually does not interfere with the normal diurnal variation and fevers are likely to be highest in the evening hours and lowest in the morning There are several other types of fever curve that maintain enough regularity in pattern to allow classification

When body temperature remains more or less continuously elevated throughout the 24 hours of the day the patient is said to have a *continuous* or a *sustained* fever This type of fever is characteristic of typhoid pneumococcal pneumonia rickettsial infections and certain viral diseases such as psittacosis

An *intermittent* or *quotidian* fever shows daily elevations with return of the temperature curve to the normal base line at least once during each 24 hour period If the patient s fever shows variations of more than 1 F during the 24 hour period of the day but does not return to the base line it is

The studies of Collins and Wood, Berlin and Wood and Kaiser and Wood have led to further understanding of the mechanism of production or release of leukocytic pyrogen The pyrogen is actively produced by leukocytes, and under appropriate environmental conditions in vitro leukocytes will continue to discharge increasing amounts of pyrogen into the suspending medium for several hours The production of this pyrogenic material involves active metabolic processes *within the cell* and *its release from the cell* is dependent on appropriate concentrations of electrolytes particularly potassium The pyrogen itself has not yet been completely purified but according to Rafter it appears to be a protein The inflammatory response apparently activates polymorphonuclear leukocytes to produce and release pyrogen Incubation of endotoxin and polymorphonuclear leukocytes in vitro results in production of leukocytic pyrogen

There is a degree of species specificity for both leukocytic and serum endogenous pyrogens This is not absolute but for example human leukocytes will not produce fever in rabbits The failure of investigators in the past to observe pyrogenic activity when inflammatory exudates of human origin were tested in laboratory animals is perhaps explainable on this basis

Endogenous Pyrogens in Man The best confirmation of a role of endogenous pyrogenic substances in the pathogenesis of fever in man comes from Snell s studies of sterile exudates from patients with fever With few exceptions the reinjection at a later time of these materials into the patient produced a sharp febrile response The pyrogenic activity was heat labile and it disappeared from body fluids with the subsidence of the pathologic process responsible for the fever Thus far attempts to demonstrate a pyrogen in human blood have been unsuccessful but it is possible that this failure may be related to dosage Indeed in animal experiments the detection of endogenous pyrogen in serum is sometimes difficult and requires the use of large volumes in testing

Steroid Fever Kappas and his associates have shown that a certain number of steroid hormones are pyrogenic when injected intramuscularly in man and there is some evidence for a role of these substances in rare instances of unexplained long-continued fever However, these materials produce no fever in laboratory animals and it is doubtful that they play a role in fever accompanying infectious disease Shulman has suggested that their pyrogenic action (which is evident only after *intramuscular* administration and *not after intravenous infusion in man*) may be secondary to extensive inflammation at the injection site

The Role of Endotoxin in Fever

An obvious possibility recognized for a long time is a role of endotoxin in the pathogenesis of the fever produced by gram negative organisms Inconclusive results were obtained in early studies conducted on the assumption that convalescence from a gram negative bacillary infection should be accompanied by a demonstrable tolerance to the pyrogenic action of endotoxin if the endotoxin were responsible for the fever of the disease The recent studies by Greisman and Hornick on the pathogenesis of typhoid fever in volunteers have clarified the situation considerably Typhoid confers tolerance to the pyrogenic effect of the endotoxin of the typhoid bacillus and to endotoxins from other species This tolerance is *demonstrable during convalescence* but not during the active phase of disease Indeed during active infection there is a hyperactivity to injected endotoxin (of homologous or heterologous origin) Presumably the patient with typhoid is exposed to a constant infusion of endotoxin and the effects of this type of exposure may be very different from those seen with *intermittent* injections Even in volunteers made tolerant by daily injections of endotoxin before the induction of typhoid fever *tolerance disappears* during the active infection but reappears immediately when disease is terminated by chemotherapy *Further studies* of these patients by Greisman and Wagner have shown that tolerance to the pyrogenic effect of endotoxin is *accompanied by an increased* ability to clear the bloodstream of endotoxin and that this increased clearance is specific *for endotoxins and does not apply* to other materials such as colloidal gold etc

Unequivocal tolerance to endotoxin has also been demonstrated by McCabe in ani

diseases may be explained by some ancillary protective effect of these illnesses against infection by the herpes virus Certainly the factors underlying the differential incidence of herpes labialis in various febrile diseases could be explored profitably

Fever alone is capable of producing enough disturbance in cerebral function to result in *delirium* Individual susceptibility to this complication of fever is quite variable preexisting disease of the brain (cerebral arteriosclerosis alcoholism) increases the likelihood of febrile delirium Disorientation is particularly common in typhoid psittacosis and rickettsial disease

A rather common complication of febrile illness in children is a *convulsion* While children with a family history of idiopathic epilepsy are somewhat more susceptible to febrile convulsions these do not necessarily herald the onset of epilepsy or some serious disorder of the nervous system

Ordinarily elevation of body temperature is accompanied by a proportional increase in pulse rate *Relative bradycardia* is seen in typhoid fever tularemia and psittacosis but it is by no means limited to these infections Individuals with acute meningitis may show impressive slowing of the pulse presumably as a result of increase in intracranial pressure A disproportionate bradycardia is also observed with considerable frequency in infectious mononucleosis mumps dengue primary atypical pneumonia and yellow fever (Faget s sign)

Patients with febrile disease often complain of *headache* but this is not invariable and fever itself is probably not responsible for the cephalgia Studies of the headache accompanying fever induced by the intravenous injection of typhoid vaccine have indicated that the pain is a result of dilatation and stretching of the large arterial vessels at the base of the brain similar to that seen in histamine headache It has also been shown that if one increases the cerebrospinal fluid pressure the intensity of headache is reduced

Whether fever alone is actually responsible for physical discomfort is an extremely *difficult question It is well known that* some patients with tuberculosis feel surprisingly well in the presence of high fever Indeed this characteristic of the febrile response in tuberculosis has been referred to as spes phthisica The fact that antipyretic drugs are also analgesics makes it very difficult to separate the symptoms of elevation of body temperature from the discomfort accompanying invasion of the body by the responsible infectious agent

Fever as a Protective Mechanism

Fever has sometimes been regarded as one of the body s protective responses to invasion by microorganisms The wisdom of using antipyretic drugs to reduce fever has been questioned from time to time on this basis Furthermore induction of fever by artificial means has been shown to have great therapeutic benefit in some diseases including syphilis of the central nervous system ophthalmic inflammations and certain types of arthritis

An extensive review by Bennett and Nicastri of the available evidence both experimental and clinical failed completely to give any support to the concept that elevation of body temperature per se exerts a protective effect or plays any important part in host defense On the other hand the harmful effects of hyperthermia are well substantiated and it is obvious that the increased demands on the heart accompanying elevation of body temperature may exert a deleterious effect in individuals with heart disease Therefore there is little question about the advisability of reducing body temperature in many patients with fever

CIRCULATORY CHANGES

Many hemodynamic changes observed in animals or patients with infectious disease are directly related to changes in body temperature Others result from direct damage to the heart the pericardium the vessels or the central nervous system by the infecting agent or its products When myocardial damage is already present high fever or the stress of infection may lead to cardiac decompensation For example individuals with mitral stenosis sometimes develop heart failure in the course of relatively trivial viral respiratory illness Whether these infections exert their seemingly dispropor

referred to as a *remittent* fever Very wide swings of temperature of a remittent or intermittent pattern constitute a *hectic* or *septic* fever Remittent fevers are characteristic of patients with brucellosis, and the fever of typhoid is sometimes mildly remittent rather than sustained

Of course hectic fevers are characteristic of pyogenic infections (particularly when there is localized suppuration) tuberculosis and malaria A hectic temperature curve which shows 2 distinct spikes during the 24 hours of the day is referred to as a *double quotidian* curve and is a classic sign of gonococcal endocarditis or kala azar At the present time in the United States a double quotidian curve is more suggestive of miliary tuberculosis than anything else although many of these patients will show the pattern for only a few days a a time

Occasionally one sees a hectic temperature curve in which the normal diurnal gradient seems to have been reversed The patient will show high spiking fever in the morning hours This pattern so-called typhus inversus is highly suggestive of tuberculosis

Recurrent or *relapsing* fevers consist of short febrile episodes of a few days interspersed with similar periods of normal temperature Rat bite fever cause by *Spirillum minus* relapsing fever (*Borrelia*) malaria and chronic menigococcemia commonly elicit this type of febrile response Recurrent fevers are not infrequent in patients with tuberculosis and are also seen in the type of brucellosis which occurs in the Mediterranean area (hence the name undulant fever) although brucellosis in the United States is accompanied by an intermittent or mildly remittent fever in most instances

Many viral diseases are biphasic and are characterized by a few days of febrile illness apparent recovery and then a second period of illness and fever The minor illness and the major illness of poliomyelitis exemplify this type of pattern Infections by the Coxsackie virus dengue smallpox yellow fever Colorado tick fever lymphocytic choriomeningitis and infectious mononucleosis are other viral or presumed viral infections in which there may be two rather distinct periods of febrile illness separated by a few days of well being

Rigors For reasons that are incompletely understood shaking chills are characteristic of certain infections and extremely rare in others In evaluating the significance of chills it is important for the physician to remember that the administration of antipyretic drugs such as aspirin can induce chills in patients with fever of almost any type and it is necessary to differentiate chilliness, chilly sensations and true chills Repeated chills suggest pyogenic infection with intermittent discharge of bacteria into the bloodstream such as can occur in infections of the liver the biliary tract and the kidneys Malaria of course is characterized by chills at rather regular intervals A single chill in a patient with bacterial pneumonia suggests pneumococcal etiology, and the occurrence of repeated chills is strong evidence that the pneumonia is caused by some other organism

Chills are quite rare in certain diseases The onset of typhoid is usually unaccompanied by chill and rigors are unusual in uncomplicated viral hepatitis Therefore in a patient with jaundice the presence of true chills is strong evidence for leptospiral infection or ascending cholangitis Repeated chills would also be good evidence for a diagnosis of bacterial endocarditis or bacterial pneumonia rather than rheumatic fever or pulmonary infarct

Other Accompaniments of Fever Fever often activates the herpes simplex virus resulting in the production of so-called *fever blisters* Some infections are more likely to be complicated by herpes labialis than are others Fever blisters are very common in pneumococcal infections malaria, rickettsioses meningoccemia and streptococcal infections especially scarlet fever In contrast, the presence of herpes labialis is strong argument against a diagnosis of primary atypical pneumonia typhoid, tuberculosis smallpox brucellosis or staphylococcal disease

The fact that artificial fever therapy results in the appearance of fever blisters in almost half of patients undergoing this type of treatment seems to indicate that it is the elevation of body temperature itself that activates the virus Therefore the rarity of fever blisters in certain febrile infectious

diseases may be explained by some ancillary protective effect of these illnesses against infection by the herpes virus Certainly the factors underlying the differential incidence of herpes labialis in various febrile diseases could be explored profitably

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tionate effect on the circulation by aggravating the retention of sodium or by direct action on the myocardium is not known However it is clear that support of myocardial function becomes a major therapeutic objective when such pre-existing lesions complicate the course of infectious disease

In this section no attempt will be made to categorize all possible circulatory changes associated with specific infectious diseases Rather the major types of alteration in body hemodynamics will be discussed and selected examples described

Central Circulatory Failure

Circulatory failure may be central or peripheral Central circulatory failure is the term used to describe any impairment of the heart as a pump which results in a cardiac output that is inadequate for the body s needs It may be the result of damage to heart valves as can occur in the course of subacute bacterial endocarditis It may be the result of myocarditis of viral or rickettsial origin The heart is also directly affected by diphtheria toxin and by severe bacterial infections such as meningococcemia Heart failure may be the result of pericardial disease with the development of acute tamponade as can occur in acute purulent pericarditis or the gradual development of heart failure as is seen in chronic tuberculous pericarditis

It seems to be unnecessary here to rehearse the pathogenesis of the clinical manifestations of congestive heart failure When evidence of impairment of myocardial function occurs in the course of infectious disease the therapeutic measures employed differ in no important way from those utilized in individuals without infectious disease The presence of known heart disease of any type should alert the clinician to the possibility of incipient congestive heart failure

Bacterial infections of the lung are often associated with transient bouts of cardiac arrhythmia such as atrial fibrillation These arrhythmias usually disappear as the infection is brought under control and do not necessarily indicate pre-existing heart disease or significant myocardial damage

Electrocardiographic abnormalities can be detected with considerable regularity in a number of common infectious diseases including viral diseases such as poliomyelitis mumps measles etc but there is little evidence that these transient abnormalities are of immediate or long term physiologic significance However, the potential seriousness of otherwise banal infections in terms of possible damage to the myocardium must always be borne in mind by the clinician

Peripheral Circulatory Failure

The picture of peripheral circulatory failure or shock with severe hypotension tachycardia cold clammy extremities evidence of cerebral ischemia thirst etc can be elicited by several mechanisms In most instances of infectious disease shock probably results from a combination of several physiologic alterations which lead to a reduction in the circulating blood volume or extensive local or general vasodilatation

In any severe systemic disease irreversible damage to body cells by interference with metabolism or by toxic injury can result in a drop in blood pressure and peripheral circulatory failure When this type of shock appears in an individual with an active infectious disease the only lasting therapy is that directed toward elimination of the infectious agent or toward neutralization of toxic products of the offending microorganisms While measures to support the circulation are indicated they will inevitably fail if employed without specific attention to the infectious agent responsible for the damage to body cells Because damage to cells may be irreversible specific therapy of an infection with antibiotics or by appropriate surgery may fail to modify peripheral circulatory failure and patients may go on to die despite the achievement of a bacteriologic cure This is seen occasionally in patients with bacterial meningitis and particularly in individuals with pneumonic plague

Reduction in blood volume in an infectious disease may result from dehydration a frequent event in very young or very elderly patients Severe diarrhea or vomiting causes increased fluid loss and in many ill individuals dehydration develops as a result of failure of the individual to maintain an intake of fluids through anorexia unwillingness to cooperate or neglect

In disease produced by meningococci various rickettsiae and certain viruses responsible for the so-called hemorrhagic fevers there is severe damage to capillaries and venules with transudation of electrolytes water and plasma proteins into the extravascular tissues These patients may develop peripheral circulatory failure as a result of the reduction in circulating blood volume despite the presence of edema

Defervescence, Hypotension and Hyperthermia. A rapid rise in body temperature such as may occur with a shaking chill involves massive autonomic activity of the adrenergic type In an individual with rising temperature there is extreme peripheral vasoconstriction with a grayish pallor there may be elevation of blood pressure agitation and pupillary dilatation Perhaps more important as a source of clinical confusion are the changes accompanying rapid defervescence In this situation there is extreme cholinergic activity with peripheral vasodilatation diaphoresis and other changes such as pupillary constriction and involuntary urination With any intense activity of the mechanisms for dissipation of heat there is very likely to be a drop in both systolic and diastolic blood pressure Patients undergoing rapid defervescence may be uncomfortable because of profuse sweating but the hypotension that so regularly accompanies this heat loss is not ordinarily dangerous and certainly does not signify failure of the peripheral circulation Hypotension is seen regularly after a paroxysm in malaria it is a frequent accompaniment of spontaneous crises is pneumococcal pneumonia and it occurs with regularity following single chills in individuals with transient bacteremia

If defervescence is induced by drugs such as aspirin there may be considerable drop in blood pressure a striking example of this being the picture that occurs when individuals with typhoid fever are given an antipyretic drug The administration of cortisone with rapid defervescence can be followed by hypotension which may last for several hours Similarly in individuals whose defervescence is the result of a dramatic response to the administration of antibiotics there may be moderate hypotension for as long as a day or two

With the exception of occasional individuals with pre existing hypertension the drop in blood pressure accompanying defervescence rarely has any serious result although it is certainly preferable to avoid too rapid cooling in febrile patients whenever possible However it is most important to avoid confusing this type of transient hypotension with true peripheral circulatory collapse

The Role of Endotoxin in Shock. Among the many potent biologic effects of the endotoxins elaborated by gram negative bacteria is their ability to produce hypotension and eventually severe circulatory failure Before discussing a possible role of endotoxin in the shock associated with various infectious disease it should be mentioned that there is some evidence to support the idea that these substances have a role in shock of non infectious origin The possibility that the irreversibility of hemorrhagic shock is a result of absorption of preformed endotoxin from the intestinal lumen has been explored extensively and has not yet been excluded However a discussion of the role of endotoxin in the pathogenesis of noninfectious shock is not germane at this point and the reader is referred to the publications of Fine in this connection

Gilbert has reviewed the mechanisms of the circulatory changes produced by endotoxin The available information is rather difficult to interpret in view of the fact that injection of endotoxin produces a different sequence of changes in various animal species Therefore as in other areas utmost caution must be exercised in applying the findings in various animals to man However despite the marked species differences several things can be said about the effect of endotoxin on the circulatory system

The effect of endotoxin is not limited to a single portion of the cardiovascular system Following injection of endotoxin in most species there is a stage of intense increase in total peripheral resistance which with large doses is followed by a later fall as vasodilatation occurs This sequence of changes fits direct observations on the effect of endotoxin on the reactivity of small vessels to epinephrine With low doses of endotoxin there is sustained vasoconstriction

with moderate doses there is vasoconstriction followed by dilatation and with lethal *doses vasodilatation may be the only re* action observed The fact that peripheral resistance is quite high in the initial stages of endotoxin shock fits the observation that the administration of vasodilators prior to endotoxin is protective and that vasocon strictors have no such effect

Profound hypotension is a part of the reaction to large doses of endotoxin in every species studied This appears to result from impairment of venous return to the heart and the decreased venous return is doubt less a result of pooling of blood in capillaries and veins Among the striking species dif ferences in the effect of endotoxin is the location of this pooling For example there is entrapment in the liver and other abdom inal viscera of the dog However, the changes *in the dog* are not seen in the monkey the cat or in man In these last three, it cannot yet be stated with certainty where the major portion of the pooling occurs However it is clear that the picture of endotoxin shock in man and other animals is not attributable to any reduction in total blood volume but *is a result of a redistribution of blood within* the intravascular space

The relative roles of catechol amines sero tonin histamine and other vasoactive agents known to be released into the circulation after the injection of endotoxin are unknown at the present time

Cortisone and related steroids are clearly capable of modifying the reaction of animals to endotoxin particularly when these hor mones are administered beforehand but *there is very little evidence that the cir*culatory failure produced by endotoxin is a result of adrenal insufficiency Indeed the circulating levels of these steroids are elevated in *individuals with reversible endo* toxin shock and it is possible also to demon strate increased peripheral utilization of injected steroids In contrast in fatal endo toxin shock there is evidence that the high levels of circulating hydrocortisone result partly from an impairment of peripheral *utilization*

A possible exception to the statement that endotoxin does not produce its effects through adrenal insufficiency is the occa sional occurrence of massive bilateral adrenal hemorrhage (Waterhouse Friderichsen syn drome) in individuals with certain types of bacteremia While adrenal apoplexy is not limited to patients with gram negative in fections it is far more frequent with this group and its relationship to endotoxin and to shock seems to be well established

HEMATOLOGIC CHANGES

Infectious diseases of many types are accompanied by changes in the formed elements of the blood These alterations include variation in the number and the type of circulating leukocytes, anemia ac *celeration of the sedimentation rate* of erythrocytes and alterations in the numbers of circulating platelets None of these hema tologic abnormalities is peculiar to infectious disease the situation being similar *to that* for the other systemic manifestations dis cussed in this chapter Despite an enormous body of clinical information concerning the alterations to be expected in specific in fections of one type or another our knowl edge of the mechanisms responsible for *many of the changes is scant*

Changes in Leukocytes

The characteristic finding in most in stances of infection by pyogenic bacteria is a polymorphonuclear leukocytosis The neu trophilic leukocytes are of great importance in the initial stages of the acute inflammatory response and it is highly probable that the increase in the number of these cells in the circulation is related in some way to the *inflammatory response* A number of substances have been described which are thought to influence the level of circulating polymorphonuclear leukocytes but at the *present time, their status remains* in doubt

In recent years, the dynamics of pro duction and distribution of polymorpho nuclear leukocytes have been studied by radioactive isotopic technics Athens has reviewed the current status of leukokinetics in an excellent monograph Polymorphonuclear leukocytes are produced continuously in the bone marrow and mature granulocytes are stored in a medullary pool before entry into the circulation Further there are two

pools of granulocytes in the circulating blood and these are of approximately equal size The first pool consists of cells which are actively circulating and the second consists of granulocytes which are sequestered or marginated in various capillary beds Acute changes in the number of circulating granulocytes following such stimuli as exercise or the injection of catechol amines are apparently a result of entry of these marginated leukocytes into the circulating pool

Other stimuli such as endotoxin and presumably active infection may cause an initial increase in the number of granulocytes in the marginated pool and hence transient neutropenia but this is followed by a striking increase in total leukocytes in the circulation This increase arises from reentry of cells from the marginated pool and the addition of new cells from the stores in the bone marrow

As yet the influence of infectious disease on the rate of production and maturation of granulocytes has not been carefully studied but probably there is stimulation of production and more rapid maturation It is highly probable that leukocytosis in infections is attributable initially to mobilization of granulocytes from the pools and that the participation of the bone marrow is reflected later in the eventual appearance of less mature forms of granulocytes

There are certain bacterial infections such as typhoid fever in which leukopenia is an almost constant finding at some stage of the disease In many other infections such as brucellosis tularemia and viral diseases (particularly in the early stages) including measles dengue Colorado tick fever smallpox and influenza a significant reduction in the total number of circulating leukocytes is seen with considerable frequency In almost any type of overwhelming infection there may be a reduction in the total peripheral leukocyte count. It cannot be stated with certainty what accounts for the leukopenia characteristic of these diseases

One particular type of leukopenia is of considerable significance The injection of bacterial endotoxin or of suspensions of bacteria is followed immediately by a sharp reduction in circulating granulocytes Within 30 minutes to 4 hours there is observed a rebound leukocytosis and this appears to be a result of release of cells from the marginated pool and the bone marrow reservoir

The initial leukopenia probably results from margination and sequestering of leukocytes in capillary beds particularly in the lung Clinically this transient reduction and elevation in peripheral granulocytes may be important in that misleadingly low counts will result if blood samples are taken immediately following a shaking chill and misleadingly high counts will be found if samples are taken during the rebound 4 or 5 hours after a chill

As is evident from the discussion above almost all studies on alterations in numbers of granulocytes during infections have dealt with cells in the blood Such studies may have limited significance since the polymorphonuclear leukocyte carries out its function for the most part after leaving the circulation Technics have not yet been devised for measuring the concentration and the life span of granulocytes in tissues

Eosinophilia, relative or absolute may be observed in the course of recovery from infections of several types It is common in parasitic infestations and is also likely to be seen in any individual with extensive skin rash The mechanisms which lead to blood or tissue eosinophilia are unknown although the association of these cells with hypersensitivity reactions is probably important Supravital studies indicate an almost selective avidity of the cells for the ingestion of antigen antibody complexes Almost nothing is known about the factors controlling production of eosinophils by the bone marrow their entry into the bloodstream etc

The lymphocytes of the circulating blood continue to pose a great mystery in terms of function It is generally agreed that they play some part in antibody formation and it is highly probable that certain lymphocytes at least, are capable or undergoing transformation into macrophages at sites of inflammation A large majority of lymphocytes appear to have a very long life span although one small population exhibits a much shorter period of viability Virtually nothing is known of the stimuli which lead to the appearance of lymphocytosis in the course of certain infectious diseases Absolute lym

with moderate doses there is vasoconstriction followed by dilatation and with lethal doses vasodilatation may be the only reaction observed The fact that peripheral resistance is quite high in the initial stages of endotoxin shock fits the observation that the administration of vasodilators prior to endotoxin is protective and that vasoconstrictors have no such effect

Profound hypotension is a part of the reaction to large doses of endotoxin in every species studied This appears to result from impairment of venous return to the heart and the decreased venous return is, doubtless a result of pooling of blood in capillaries and veins Among the striking species differences in the effect of endotoxin is the location of this pooling For example there is entrapment in the liver and other abdominal viscera of the dog However, the changes in the dog are not seen in the monkey the cat or in man In these last three it cannot yet be stated with certainty where the major portion of the pooling occurs However it is clear that the picture of endotoxin shock in man and other animals is not attributable to any reduction in total blood volume but is a result of a redistribution of blood within the intravascular space

The relative roles of catechol amines serotonin histamine and other vasoactive agents known to be released into the circulation after the injection of endotoxin are unknown at the present time

Cortisone and related steroids are clearly capable of modifying the reaction of animals to endotoxin, particularly when these hormones are administered beforehand but there is very little evidence that the circulatory failure produced by endotoxin is a result of adrenal insufficiency Indeed the circulating levels of these steroids are elevated in individuals with reversible endotoxin shock and it is possible also to demonstrate increased peripheral utilization of injected steroids In contrast in fatal endotoxin shock there is evidence that the high levels of circulating hydrocortisone result partly from an impairment of peripheral utilization

A possible exception to the statement that endotoxin does not produce its effects through adrenal insufficiency is the occasional occurrence of massive bilateral adrenal hemorrhage (Waterhouse Friderichsen syndrome) in individuals with certain types of bacteremia While adrenal apoplexy is not limited to patients with gram negative infections, it is far more frequent with this group and its relationship to endotoxin and to shock seems to be well established

HEMATOLOGIC CHANGES

Infectious diseases of many types are accompanied by changes in the formed elements of the blood These alterations include variation in the number and the type of circulating leukocytes, anemia acceleration of the sedimentation rate of erythrocytes, and alterations in the numbers of circulating platelets None of these hematologic abnormalities is peculiar to infectious disease the situation being similar to that for the other systemic manifestations discussed in this chapter Despite an enormous body of clinical information concerning the alterations to be expected in specific infections of one type or another our knowledge of the mechanisms responsible for many of the changes is scant

Changes in Leukocytes

The characteristic finding in most instances of infection by pyogenic bacteria is a polymorphonuclear leukocytosis The neutrophilic leukocytes are of great importance in the initial stages of the acute inflammatory response, and it is highly probable that the increase in the number of these cells in the circulation is related in some way to the inflammatory response A number of substances have been described which are thought to influence the level of circulating polymorphonuclear leukocytes but at the present time their status remains in doubt

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a result of involvement of the spleen in these diseases and occasionally the anemia has been relieved by splenectomy

As has been mentioned chronic infections of several types may be accompanied by anemia This is commonly referred to as secondary anemia, but a preferable term according to Wintrobe is simple chronic anemia There is very little alteration in the morphology of erythrocytes in this anemia and it bears a close resemblance to that observed in chronic debilitating disease of several types including disseminated tumors and chronic renal failure

Among infections in which anemia may become very prominent is subacute bacterial endocarditis In the vast majority of instances the anemia is only moderate and is not a serious clinical problem Brucellosis tuberculosis prolonged suppuration etc are also regularly accompanied by mild anemia The decrease in circulating erythrocytes in chronic infection apparently results from a combination of depression of the bone marrow and a shortened life span of the red cells One of the earliest changes noted in infection is a reduction in plasma iron content and in serum iron binding capacity This cannot be influenced by the administration of iron whether given in the usual form or as transfused erythrocytes There is delay in the incorporation of labeled iron into erythrocytes and this is mirrored by the clinical observation that individuals with chronic infection are unable to respond to such stimuli as bleeding or an acute hemolytic episode with an outpouring or reticulocytes Indeed the response of patients with pernicious anemia to therapy with vitamin B_1 may be interrupted by an intercurrent infection Other chemical changes observed in the course of the anemia of chronic infection include elevation of serum copper elevation of erythrocyte protoporphyrin and an increased excretion of coproporphyrin There is usually no evidence of increased hemolysis in the form of elevation of urobilinogen or of serum bilirubin but measurement of the life span of erythrocytes in chronic infection has regularly demonstrated a shortening At the present time the only definitive treatment for the anemia of chronic infection is control of the infectious process itself With convalescence there is prompt correction in the hematologic defect

Changes in Platelets

Thrombocytopenia has been observed in a large number of infectious diseases particularly those accompanied by bacteremia The mechanism of the reduction in circulating platelets is not known but may be sequestration in areas where there has been damage to endothelium Purpura is a relatively common manifestation of severe bacterial infections such as meningococcemia and bacterial endocarditis but the relative role of thrombocytopenia capillary fragility and other factors in these conditions remains to be worked out

A lowered number of platelets although not a reduction to levels that might be expected to lead to significant bleeding is observed with great regularity when counts are made in patients with many different types of infectious disease including typhoid the rickettsioses the viral exanthemata, subacute bacterial endocarditis and infectious hepatitis

Whether the ability of the bone marrow to produce platelets is actually impaired in the course of any of these infections is a question which has not been investigated It is probable that occasional instances of secondary thrombocytopenic purpura are attributable to hypersplenism in the course of chronic infections but this seems to be rare

WEAKNESS AND MALAISE

Patients with acute or chronic infectious disease complain of a variety of unpleasant somatic sensations These include weakness fatigability anorexia generalized aching etc While a patient may attempt to describe his symptoms in many ways one ordinarily has only to look at him to realize that he is miserable and uncomfortable beyond his ability to vocalize or to localize the seat of his complaints

Despite the great frequency of these ill defined manifestations of illness surprisingly little is known about the basic mechanisms involved The manifestations are in no way peculiar to infection and indeed many of

phocytosis of a mild degree is seen characteristically in the later stages of certain common viral diseases such as mumps and measles For mysterious reasons an enormous lymphocytosis is characteristic of pertussis In this disease the lymphocytosis appears in the early stages of infection and persists well into convalescence Finally a peculiar disease acute infectious lymphocytosis, presumably of viral origin, is seen primarily in children and is characterized by enormous increases in the peripheral count of mature lymphocytes

There are several other diseases in which lymphocytosis is seen with regularity and in which the increase in lymphocytes is attributable to atypical cells The best example of this is infectious mononucleosis a disease of presumed but not proved viral origin Similar cells are seen in other viral diseases particularly in infectious hepatitis Indeed these atypical lymphocytes are often referred to as virocytes This change in morphology remains unexplained Similar alterations have been described in response to stress but the significance of this for viral diseases is doubtful

Monocytosis has been noted with considerable regularity during the period of convalescence from many acute bacterial infections Monocytosis is sometimes especially prominent in patients with tuberculosis subacute bacterial endocarditis or listeria infections and occasionally it is seen in typhoid or brucellosis The mechanism of these fluctuations in circulating monocytes is entirely unknown and other than to point out their occurrence little else can be said

Changes in Erythrocytes

Perhaps the most frequent change involving circulating erythrocytes in the course of infectious diseases is an increase in their *sedimentation rate* While a number of factors are known to influence the rate at which erythrocytes will settle from plasma it cannot be said with certainty which of these are involved in the acceleration observed in infectious diseases The addition of fibrinogen bacterial polysaccharides and of various plasma globulin fractions will increase erythrocyte sedimentation Albumin appears to inhibit sedimentation and, interestingly enough gamma globulins are less effective than alpha and beta globulins in accelerating the sedimentation rate Further more it appears that the proportion of components of plasma protein is more important than the absolute concentration of any single one The rate of erythrocyte sedimentation is accelerated in the vast majority of infectious diseases particularly systemic infections Of course, high sedimentation rates also occur in noninfectious diseases The clinical usefulness of the test lies in its ability to indicate organic illness and to substantiate subsidence of infection or other inflammatory disease

Anemia is a common complication of infection It is seen more frequently in chronic prolonged infectious diseases than in acute bacterial and viral disorders

Acute hemolytic anemia may complicate the course of viral infections including acute viral hepatitis and herpes simplex Primary atypical pneumonia due to mycoplasma infection may be complicated by hemolytic anemia the mechanism presumably being related to the frequent development of cold agglutinins in this disease

Excepting Oroya fever the bacterial infection in which acute hemolytic anemia is seen with greatest regularity is *Clostridium welchii* bacteremia There is massive destruction of red cells hemoglobinuria and severe prostration Not infrequently control of the bacterial infection is to no avail the patient finally dying as a result of the renal complications of the massive hemolysis Despite the fact that several other bacteria are known to produce hemolysins there is little evidence except in the case of the Welch bacillus that these hemolysins are particularly active in the course of infection in man However hemolysis of significant degree has been observed in typhoid (it is probably a more regular occurrence than has been recognized previously) and rarely in beta hemolytic streptococcal infections and in infections by the influenza bacillus

Occasional instances of hemolytic anemia have been reported to complicate the course of tuberculosis and other infectious granulomata such as those produced by fungi In several instances this has appeared to be

a result of involvement of the spleen in these diseases and occasionally the anemia has been relieved by splenectomy

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Despite the great frequency of these ill defined manifestations of illness surprisingly little is known about the basic mechanisms involved The manifestations are in no way peculiar to infection and indeed many of

them occur in endocrinopathies and even in purely psychiatric disorders The present discussion will be confined to certain aspects of muscle function and to problems of convalescence

Muscle Weakness in Infectious Diseases

When the term weakness is used by a patient to describe his symptoms it is necessary to make further inquiry and to differentiate true loss of muscle strength from lassitude and fatigue When weakness can be demonstrated objectively it becomes important to decide whether the weakness is limited to certain muscle groups, in which case one is dealing with local *paresis* or whether the muscle weakness is generalized in which case a proper term is *asthenia* or *myasthenia* With paralysis or paresis, the possibility of viral infection of the nervous system or of postinfectious neurologic complications must be considered

In terms of the effect of the infectious process on muscle tissue itself there is a wide discrepancy between a patient s complaints and objective findings Adams Denny Brown and Pearson have summarized the available information on alterations in skeletal muscle associated with acute and chronic infectious diseases They classify the pathologic changes into 3 groups Zenker s hyaline degeneration muscle atrophy usually secondary to malnutrition and cachexia in chronic infections and focal myositis a relatively rare occurrence

Zenker's hyaline or waxy degeneration is accompanied by no definite clinical manifestations although it is found relatively frequently in severe infections The morphologic changes consist of focal swelling of muscle fibers usually with eventual involvement of the entire fiber In the beginning striations are easily visible but eventually these disappear, leaving a homogeneous eosinophilic hyaline structure which may undergo degeneration or more often is the seat of regeneration as the infectious process subsides This change can occur in typhoid where it was originally described by Zenker but it has been seen in fatal influenza in pneumococcal pneumonia and numerous other fatal diseases The changes are particularly common in the rectus abdominis muscle, but waxy degeneration is also found in the proximal muscles of the pelvis and the shoulder girdle in the diaphragm and in the larger muscles of the trunk Occasionally there is hemorrhage into the belly of the muscle, in which case there may be local pain but the process is ordinarily difficult to correlate with myalgia in the course of infectious disease When the changes are marked as Adams and his colleagues point out the aches and pains of diseases may be attributable, in part to this type of degeneration However the frequency of impaired muscle strength is far greater than is that of well-demarcated Zenker s degeneration and it appears that this type of change in muscle accounts for only a small part of the objective weakness that accompanies infectious diseases The extent of Zenker s waxy degeneration seems to correlate very well with the severity of the infectious process and lesions of this type have been found in diphtheria tuberculosis tetanus cholera typhoid fever, infectious hepatitis smallpox yellow fever and severe staphylococcal and streptococcal infections It is of considerable interest that similar lesions have been found in patients with noninfectious diseases such as disseminated malignant tumors, hepatic cirrhosis etc

Muscle Atrophy In elderly patients with debilitating disease particularly chronic infections one may see atrophy of muscle This is occasionally associated with vacuolation and edema of individual fibers and is probably a result of a combination of disuse the effect of microorganisms and their toxins and muscle atrophy secondary to the polyneuropathy of malnutrition The recognition of this type of muscle disorder is relatively easy because it is accompanied by other evidences of emaciation with disappearance of subcutaneous fat and reduction in muscle mass A characteristic clinical reation is the ridgelike contraction that can be elicited by a sharp blow This so-called *myoedema or idiomuscular contraction* has been believed to be specific for certain chronic infections such as tuberculosis but careful observations have shown that any chronic disease that impairs general nutrition can produce similar changes in muscle The reduction in muscle

strength in this type of atrophy is usually proportional to reduction in muscle size Consequently the strength of these atrophied muscles is sometimes surprisingly good

Focal Myositis Several infections produce specific and relatively characteristic changes in muscle in the form of focal myositis The muscle lesion in leptospiral infections particularly in Weil s disease is undoubtedly responsible for the severe myalgia that characterizes this disease There is very good evidence that it is produced by leptospiral infection of the muscle itself

While it is entirely possible for pyogenic bacteria to produce muscle abscesses this type of lesion is quite rare in the absence of septic embolization or penetrating injuries

The myositis produced by certain strains of clostridia is described elsewhere Myositis as a result of direct action of the infectious agent has been described in tuberculosis syphilis and actinomycosis

Finally it appears likely that inflammation of muscle can accompany certain viral infections in man although the clinical incidence of viral myositis remains to be evaluated by properly controlled studies Viruses of the Coxsackie group are capable of producing significant destruction of muscle fibers in suckling mice and hamsters It has been shown also that Arbor viruses and other neurotropic viruses produce significant muscle lesions in suckling mice but the clinical counterpart of these lesions in man remains to be determined

The occasional instances of myocarditis that complicate viral infections of many types may have their counterpart in focal lesions of skeletal muscle which are ordinarily so insignificant as to remain unobserved Certainly the fact that Coxsackie B viruses can produce fatal myocarditis in infants is of interest in this regard

Lassitude Fatigability and Problems of Convalescence

Lassitude and fatigue are terms often employed to describe the lack of energy experienced by patients with infectious or other diseases in the absence of objective signs of paralysis or myasthenia The frequency of the subjective sensations described by patients as all in 'without pep no ambition no interest being fed up lack of energy languor "listlessness weariness etc stands in striking contrast to the small body of objective knowledge concerning the mechanisms involved

Infection is a relatively infrequent cause of complaints of chronic fatigue The fact that certain low grade chronic infections may be accompanied by manifestations of lassitude and fatigue is indisputable but the frequency of entirely similar manifestations in individuals with no evidence of an infectious process makes it all the more difficult to link infection with these manifestations in causal fashion

Diseases such as brucellosis tuberculosis infectious hepatitis and certain viral infections of the respiratory tract including influenza are more likely to result in a rather prolonged period of debilitation than are other illnesses with more dramatic acute manifestations On the other hand careful study shows clearly the importance of psychological factors in the persistence of symptoms of lassitude and irritability following apparent recovery from infectious diseases In one such study 24 individuals with a history of acute brucellosis were studied several years after recovery from acute illness Eight of the patients had recovered promptly and uneventfully after subsidence of the febrile stage of the disease Among the remaining patients there were 6 who had remained symptomatic for a year or longer after the acute stage of brucellosis but who had subsequently recovered and 10 patients who had had persistent symptoms for 2 to 8 years since the acute febrile stage of brucellosis and were still symptomatic Medically there was absolutely no evidence of any persistent infection Furthermore extensive psychological testing revealed normal intellectual function without evidence of organic brain damage in all subjects However when these patients were evaluated independently by psychological testing and psychiatric interview it became evident that the chronically ill" patient uniformly revealed evidence of emotional disturbance usually in the pattern of depression Indeed a review of the psychological and psychiatric data made it possible for independent observers to classify each patient without prior knowledge of the

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measles vaccine was followed by varying periods of cutaneous nonreactivity with a gradual reappearance of the ability to show a positive skin test

CONCLUSION

This summary of some of the systemic manifestations to infectious diseases has laid particular emphasis on the reactions of man to invasion by pathogenic microorganisms The great frequency of infectious diseases and the manifestations of illness that have been mentioned in this chapter stand in striking contrast to the relative paucity of information about the physiologic alterations underlying both the objective and the subjective changes that are common to many infections There is perhaps no more fruitful field for future investigation than the re-examination of these common responses with a view of gaining some understanding of the basic mechanisms responsible for them and hopefully the development of methods for their control

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clinical course of his disease Thus the emotional disturbance as manifested by a chronic symptomatology in these individuals was not entirely a result of the bacterial disease but was closely related to the personality structure of the individual at the time of the acute illness

A prospective study carried out by obtaining psychological information on a closed population before the onset of an epidemic of viral influenza revealed that the *occurrence of prolonged convalescence could* be predicted Among a group of 600 individuals on whom data were obtained there were 26 documented instances of influenzal infection The symptoms of acute influenza were the same in all patients However 14 of them reported complete recovery within 14 days whereas in the remaining 12 symptoms of tiredness weakness insomnia headache anorexia etc persisted for more than 3 weeks Every individual in whom symptoms persisted showed a pattern of response in the psychological tests characteristic of depression prone individuals Since the psychological data were obtained prior to illness the emotional state or attitude was not secondary to infection but existed prior to the onset of acute illness and was a most significant factor in delaying symptomatic recovery (Imboden)

Miscellaneous Changes Associated With Infectious Disease

A variety of systemic alterations can be mentioned briefly It is not surprising that infectious diseases both acute and chronic, may interfere with general nutrition due to both reduced intake of food and increased metabolic rate Even in individuals with localized suppuration there may be striking weight loss Loss of 25 to 40 pounds within a period of 4 weeks by patients with pleural empyema or chronic lung abscess is not unusual

Acute changes in electrolytes may occur in many infectious diseases In pneumococcal pneumonia and in *tuberculosis, there is depression* in the urinary excretion of sodium and of chloride and there may be a reduction in the serum concentration of these ions Especially in individuals with tuberculosis the level of serum sodium seems to be low and despite administration of large amounts of fluid and electrolyte it is not possible to restore serum levels to the normal range for healthy individuals With response to specific antimicrobial therapy the patient s electrolyte pattern promptly returns to normal

It is not surprising that acute infections stimulate adrenal cortical function the increase being mediated through the pituitary There may also be a mild increase in thyroid function concomitant with the increase in *metabolism that occurs with elevation of* body temperature

Of considerable importance is the effect of bacterial or viral infection on certain hormonal disorders Perhaps the outstanding example of this is the profound worsening of diabetes mellitus that occurs in the presence of infection The insulin requirement in these patients is enormously increased and diabetic acidosis *with coma is* more likely to be precipitated by infection than by any other single factor excepting the omission of insulin by the diabetic patient As is well known, crisis in patients with Addison s disease is often precipitated by viral or bacterial illness and in the past many patients with adrenal insufficiency succumbed to intercurrent infection At the present time, with better control of Addison s disease through the use of complete replacement therapy this is a less serious clinical problem

Finally mention may be made of the effect of certain viral diseases on the nephrotic syndome and on the ability of the skin to react to allergens Children with nephrosis often will undergo spontaneous diuresis as a result of infection with *measles or* with the virus of sandfly fever The mechanism of this induction of remission is entirely unknown but it appears to depend on a specific property of only a few viruses Individuals recovering from measles often undergo a transient period during which they will not react to the subcutaneous injection of tuberculin or of other substances such as *codeine This period of cutaneous* "anergy is variable but may last for several months That it is closely related to infection by the measles virus is indicated by recent studies in volunteers known to react cutaneously to certain allergens *The administration of live*

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THE INDIGENOUS BACTERIA AND FUNGI

The following listing is limited to the more clearly recognized species found commonly in the absence of significant disease in one or more loci of the normal flora of man Pathogenic associations are noted Sites fre quencies of occurrence and concentrations are considered in a later section

Cocci

Staphylococci Among gram positive mass forming cocci the principal members of the indigenous biota are the potentially patho genic (coagulase positive) *Staph aureus* (*Staph pyogenes*) and the coagulase negative *Staph albus* Tetrad (*Gaffkya tetragena*) and packet (*Sarcina lutea*) forming cocci have also been recovered from the human mouth (Pike 1962) but less regularly *Staph albus* appears to be a typical am phibiont

ANAEROBIC MICROCOCCI The least ob scure members of this poorly studied group are *Staphylococcus anaerobius* (Foubert and Douglas 1948) a nonfermenting catalase former and *Micrococcus niger* which forms gas and a black pigment

Streptococci Group A *Str pyogenes* and *Diplococcus pneumoniae* have been recovered often enough from healthy persons to be thought of as indigenous However the char acteristic indigenous streptococci fall into the enterococcus and the viridans groups

ENTEROCOCCI are characterized by the group D polysaccharide They are extraor dinarily resistant to environmental influences (see Buttiaux and Mossel 1961) Most spe cies of enterococci grow at both 10 and 45 at pH 9 6 in 6 5 per cent NaCl and in 40 per cent bile and withstand 60 temperature for 30 minutes They are generally resistant to penicillin The commonest species in man are *Str faecalis* and *Str faecium* which are distinguished by failure of the latter to reduce litmus milk or to grow in 1 2 500 potassium tellurite and of the former to ferment ara binose Varieties of *Str faecalis* which yield β hemolysis (*Str zymogenes*) or liquefy gelatin (*Str liquefaciens*) are also common (see Papavassiliou 1962 Colobert *et al* 1962 Defayolle and Colobert 1962)

Enterococci are amphibiotic in man and animals without clearly defined primary pathogenicity but are nevertheless capable of causing disease in some situations notably subacute bacterial endocarditis and urinary tract infections An attempt by Deibel and Silliker (1963) to confirm experimentally in human volunteers early suggestions that en terococci may be implicated in food poison ing was unsuccessful

VIRIDANS STREPTOCOCCI are a heterogen ous group of amphibionts principally non hemolytic sometimes greening on blood They lack either D or A polysaccharides (or the group N antigen of the lactic strepto cocci) The Lancefield polysaccharides C E F G H O K and M have been identified in streptococci that probably belong in the viridans groups Williams (1956) for ex ample found that 58 per cent of 103 strains of *Str salivarius* reacted with group K anti sera and considered this and other antigens of this class (H and E) to be nonspecific (See also Ottens and Winkler 1962 Rifkind and Cole 1962 Stewart and McKeever 1963)

Str salivarius (syn *Str hominis*) the most clearly defined viridans streptococcus is characterized by levan production and large mucoid colonies on 5 per cent sucrose agar It is usually nonhemolytic on blood agar grows at 45 but not at 10 ferments inulin salicin and esculin and acidifies and coagulates litmus milk

Str mitis The remaining members of the viridans group although various may be grouped pending more adequate classifica tion under the specific name *mitis* All are levan and inulin negative and do not grow as mucoid colonies on sucrose agar but some form dextran from sucrose These are char acteristically but not invariably greening on blood agar Streptococci of subacute endo carditis fall largely in this group but attempts to define a pathogenic species (so-called *Str sanguis* or *Str s b e*) are not considered valid The encapsulated *Streptococcus MG* used diagnostically in primary atypical pneu monia may be a distinctive antigenic type

PEPTOSTREPTOCOCCUS Anaerobic strep-tococci some of which become adapted to aerobic growth have been reclassified as a separate genus by Breed *et al* (1957) Little

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14

Bacteria Indigenous to Man

From the moment of birth and throughout life man is exposed to microorganisms The resultant effects may take a seemingly continuous range of forms depending on the distinctive reactivity of the microorganisms Many true saprophytes fail to maintain themselves on human tissues But given suitable partial adaptation of microbe to host species the resultant is likely to include a manifest host response recognizable as overt disease Pathogens may achieve equilibrium with the host either from the start (as in healthy carriers of pneumococci or hemolytic streptococci) or after an initial upset as with a wide and familiar range of infective diseases As discussed in Chapter 2 there is a continuous spectrum from the imminent or potential pathogenicity known as latency through apparently permanent neutrality (the resultant of the interaction being undetectable) to symbiosis The word commensalism is often applied to this intermediate range but like other terms that grew out of an early and necessarily rigid interpretation of the germ theory in which the focus was sharply on disease and a simple cause and effect relationship commensalism implies a passivity that now seems to be unsophisticated A better term borrowed from vertebrate zoology may be amphibiosis —including both symbiosis and antibiosis (or pathogenicity) or anything between the two

The indigenous bacterial biota of man may thus be characterized as occupying a band of a continuous spectrum between saprophytes and pathogens typically neither but merging with both In the middle of the band the biota consists of obligate parasites which in pure culture lack obvious or easily demonstrable pathogenicity Some of them are distinctive species known to occur only in this habitat The greater variety are closely related to the more characteristic freely communicable bacterial pathogens for man On the other hand animal pathogens that infect man aberrantly and are not usually communicated from man to man like *Pasteurella* or *Brucella* do not have relatives in the indigenous biota These circumstances suggest an evolutionary adaptation of both participants from pathogenicity toward amphibiosis

In this context the word infection may need redefinition All that it implies is an interaction of a host organism with a smaller self replicating agent This definition is in line with the now generally held view that infection can occur without disease which is only one of its possible consequences At the end of the spectrum in which the microorganisms concerned are most highly adapted to man as host—the true amphibionts—the relationship is a balanced and stable one Nevertheless the balance still may be upset with resulting damage to either parasite or host

The following account of the indigenous biota including the sections on its distribution and significance and on its nonpathogenic effects is abridged with additions from the more recent literature from Rosebury (1962)

fermenti with smooth to intermediate colonies less actively saccharolytic and having intermediate acidogenicity and (5) *L brevis* rough with short to medium bacilli fermenting a wider range of test carbohydrates than the preceding but producing relatively little acid

Corynebacterium These principally or exclusively catalase positive gram positive nonmotile rods often pleomorphic include the characteristic diphtheroids of mucous membranes The traditional simple criterion of fermentation of glucose and sucrose separates the nonfermenting *C hofmanni* (*C pseudodiphtheriticum*) and the fermentative *C xerosis* from pathogenic forms (*C diphtheriae* and others) which ferment only glucose However nontoxigenic *C diphtheriae* and otherwise nonpathogenic glucose fermenters are well recognized and all 3 fermentation patterns appear commonly among indigenous species *C haemolyticum* and other aerobic catalase negative forms are probably more appropriately classified as streptococci or in other genera (see Zinneman and Turner 1963)

Corynebacterium acnes and other anaerobic or micro aerophilic diphtheroidlike bacteria constitute an ill-defined group members of which have been classified variously as *Lactobacillus Actinomyces Propionibacterium* and in several less familiar genera The definition of *Lactobacillus* given above separates that group It may be feasible to include catalase negative anaerobic forms in the genus *Actinomyces* as noted below Among the remaining members of this group the catalase positive anaerobe *C acnes* (or *Propionibacterium acnes* see Moore and Cato 1963) is indigenous to skin and widely distributed in other locations both amphibiotically and saprophytically Several studies have established a clear distinction between this organism and the anaerobic actinomycetes Bouisset *et al* (1963) have reported differences in DNA base ratios between the aerobic diphtheroids and the anaerobes

Actinomyces If the generic name *Actinomyces* is limited to anaerobic catalase negative gram positive bacilli with a tendency to branch it would include 2 groups (1) those associated with actinomycosis the *bovis israelii* forms and (2) *A bifidus* more commonly called *Lactobacillus bifidus* but excluded from the latter genus by several groups of workers in recent years

A distinction between two species of the first group *A israelii* and *A bovis* now seems unequivocal on the basis of differences in cell wall composition and serology (King and Meyer 1963) *A israelii* is a rough amphibiotic form which sometimes causes actinomycosis in man *A bovis* is smoother more diphtheroidlike and is found typically in cattle

Actinomyces bifidus (*Lactobacillus bifidus*), although recognized since the turn of the century as the predominating bacterium of the feces of breast fed babies remains inadequately defined and classified Studies especially during the 1950 s which have established the characteristics of the lactobacilli generally have excluded the bifidus forms both morphologic and metabolic findings have pointed to a relationhip to *A israelii* Attempts to subdivide the bifidus group have yielded inconsistent results The occurrence of a variety *pennsylvanicus* (Gyorgy *et al* 1954) which requires for growth a mucopolysaccharide fraction of blood group substance found in human milk and colostrum has been suggested as explaining the bifidus nursling relationship but the *A bifidus* found in nursling feces has simpler nutritive requirements not including the *pennsylvanicus* factor and it is possible that human milk selects *A bifidus* by inhibiting coliforms and enterococci rather than by direct stimulation

Leptotrichia General agreement seems to have been reached in the early 1960 s that this generic name and the single identified species name *L buccalis* be limited to a bacterium long recognized as an amphibiotic resident of the human mouth a rather thick unbranched gram positive organism growing as long rods or filaments anaerobic catalase negative homolactic (final pH approximately 5 0) forming typical translucent rough colonies on enriched agar Irregularities of morphology and a tendency to decolorize in the gram stain have led to confusion between this organism and *Fusobacterium* which is however morphologically and biochemically distinct

Bacterionema matruchotii (syn *Lepto*

attention has been paid to them in recent years and they are in need of reconsideration These streptococci have been associated with endogenous anaerobic mixed infections including a form of puerperal fever studied especially in the 1930s wound and other infections in which bacteroides were also implicated and the so-called fusospirochetal infections as detailed in a later section Although additional species names are used the following represent the principal ones

P putridus a strict anaerobe gas forming and fetid forms large volumes of gas, principally CO from glucose fructose and maltose but only in the presence of S-containing compounds (Hare *et al* 1952) Also formed are NH_3 H S and formic butyric and acetic acids The cocci tend to be large and pleomorphic

P evolutus which acquires oxygen tolerance and *P micros* a strict anaerobe both fail to produce gas or odor (see Hare *et al* 1952 Mergenhagen and Scherp 1957)

Neisseria *N meningitidis* like group A streptococci and pneumococci has frequently been found in healthy carriers in whom it may achieve amphibiotic status However the more typically indigenous members of *this genus are characteristic amphibionts* The generic name should be limited to aerobic oxidase positive gram negative cocci Principal species are *N catarrhalis* unpigmented no acid from carbohydrates *N sicca* unpigmented ferments glucose maltose fructose and sucrose (but see Berger 1961a b) resembles the meningococcus but grows more abundantly and at 22° it forms a dry rough colony and is antigenically distinctive *N pharyngis* chromogenic (forming greenish yellow colonies) fermentative with strain variations antigenically heterogenous and *N mucosa* resembling *N sicca* but encapsulated mucoid and serologically distinct

Pike *et al* (1962) have suggested that nonpathogenic neisseria including named strains from various sources and fresh isolates from the mouth all could be grouped as *N pharyngis* and *N catarrhalis* of which the former was the more common For a review of nonpathogenic neisseria see Berger (1963)

Veillonella. Although several species of strictly anaerobic gram negative cocci are thought to exist only one *V alcalescens* (syn *V gazogenes Micrococcus lactilyticus*) has been identified clearly (see e g, Rogosa *et al* 1961) This widely distributed amphibiont is a small spherical mass forming coccus which may be gram positive during the first few hours of growth but is uniformly gram negative after 12 hours It fails to ferment sugars but ferments lactate and other *intermediate* products of hexose breakdown producing propionic and acetic acids with CO and H in large amounts, accompanied by a rise in pH Many biochemical studies of this organism have been published (see e g McCormick *et al* 1962a b Whiteley and McCormick 1963 Woolfolk *et al* 1962) *V alcalescens* is apparently not specifically pathogenic It contains endotoxin (Mergenhagen *et al* 1962 1963)

Gram positive Bacilli

Lactobacillus The generic name *Lactobacillus* is coming to be limited to catalase negative gram positive nonmotile rods that *grow aerobically and ferment* carbohydrates actively either homofermentatively to lactic acid or heterofermentatively to lactic acid and other products including CO (The anaerobic *L bifidus* is included under *Actinomyces* see below) Many species of lactobacilli inhabit the mucous membranes Of these only one *L acidophilus* is obligately parasitic the others including the commonest and most numerous indigenous species are widely distributed in nature Some of these organisms by virtue of their high acid production may be a significant factor in dental caries They are thought to play a symbiotic role in limiting the flora of the vagina

Among indigenous *homofermentative* lactobacilli the commonest species found are (1) *L casei* with smooth colonies and curved bacilli growing at 15° and irregularly at 45° actively acidogenic (2) *L acidophilus* rough with long bacilli growing at 45° but not at 15° less actively saccharolytic and acidogenic and (3) *L plantarum* smooth with short straight bacilli growing at 15° but usually not at 45° actively saccharolytic and acidogenic The two commonest *heterofermentative* species are (4) *L*

cific or variety names (*Bacterium* etc) *anitratum* and *glucidolytica* This organism is also known by the notebook code designation B5W

Although the literature on these microorganisms deals predominantly with their occurrence in disease they also reside on normal mucous membranes The principal sites in which members of the group have been found normally are the nose the vagina and the conjunctiva Pathologically they have been recovered from a wide range of sites and conditions The conditions attributed to them include for all 3 species conjunctivitis and meningitis and especially for penicillin resistant forms (*Mima* spp) gonorrhealike urethritis Attempts to demonstrate experimental pathogenicity have been successful in some instances but in general only with massive doses suggesting a typical amphibiotic behavior

Pseudomonas Alcaligenes A large group of gram negative strictly aerobic saprophytic bacilli some of which are pigment formers and manifest obligately aerobic glycolysis (*Pseudomonas*) while others are achromogenic and may be nonglycolytic (*Achromobacter*) are linked in part because variants of the first group may lose or fail to show the distinguishing features mentioned Motility due to polar flagella is characteristic of the first group and is variable in the second The most important strains are *P aeruginosa* (*Bacillus pyocyaneus*) and *Alcaligenes* (*Achromobacter*) *faecalis*

Pseudomonas aeruginosa is identified by the formation of water soluble fluorescent bluish green pigments (the blue green phenazine pigment pyocyanin and the greenish yellow fluorescein) It is proteolytic and forms acid from glucose only when grown with free access to air Other distinguishing features are oxidation of potassium gluconate a rapidly positive test for cytochrome oxidase and rapid breakdown of arginine Along with staphylococci *Candida albicans* and a few other species this micro-organism has become of greater pathogenic importance during the antibiotic era (see Flynn and others 1963) The increased frequency of pseudomonas disease is based at least in part on the widespread distribution of this organism and on its ability to develop resistance to antibiotics Experimental pathogenicity has been demonstrated under conditions typical for amphibionts entailing e g injection of 10^8 or more organisms intraperitoneally into mice or pretreatment with x radiation

Alcaligenes faecalis is distinguished from *Mima polymorpha* which it resembles biochemically by being consistently bacillary and gram negative growing regularly on simple nutrient agar and being frequently motile It does not attack glucose or other carbohydrates but grows in a medium containing only lactate asparagine and salts It is found in feces and appears uncommonly in a variety of pathologic situations (see Sherman *et al* 1960)

Vibrio Aerobic members of this genus recovered from feces e g *V alcaligenes* and *V percolans* have been found to be indistinguishable from *Alcaligenes faecalis* or from pseudomonads

Haemophilus Of the nutritionally fastidious aerobic gram negative bacilli requiring phosphopyridine nucleotide (V factor) or hemin (X) or both *H influenzae* and the hemolytic *H haemolyticus* which require both are characteristically found in the healthy mouth and upper respiratory tract as are the corresponding forms that require only V factor *H parainfluenzae* and *H parahaemolyticus* The indigenous influenza bacillus proper is usually rough and lacks the *b* capsular antigen of the typical pathogen for man but probably possesses endogenous pathogenicity

H aegyptius (the Koch Wells bacillus) antigenically distinct from non type specific *H influenzae* has been found in the healthy conjunctival sac as well as in conjunctivitis Two other species *H vaginalis* and *H aphrophilus* are both probably better classified in other genera Neither species appears to require X or V factors *H vaginalis* associated with vaginitis but probably indigenous to the area (see Dunkelberg *et al* 1962) has been classified as *Corynebacterium vaginale* principally on morphologic grounds but differs from diphtheroids in being catalase negative (Zinnemann and Turner 1963) *H aphrophilus* is a little studied gram negative aero

TABLE 1 PRINCIPAL DISTINGUISHING CHARACTERISTICS OF *Moraxella Mima**

Neisserialike short rods or coccal forms in pairs or short chains with some longer rods and filaments gram negative to gram amphophile aerobic anaerogenic Indole and Voges Proskauer negative Usually nonmotile encapsulated and methyl red negative

	Serum Required for Growth	Growth in Defined Media	Liquefaction of Serum or Gelatin	Acid from Glucose	H S Formed	NO_3 Reduced to NO	Oxidase	Sensitive to Penicillin
Moraxella lacunata	V	0	V	0	0v	+	+	S
Mima polymorpha	0	V	V	0	V	0v	V	V
Mima vaginicola	0	+	V	+	V	0	0	R

\+ positive 0 negative V variable 0v usually negative S sensitive R resistant

* (From Rosebury [1962] McGraw Hill Book Co)

trichia dentium) is another oral bacterium sometimes confused with *L buccalis* but clearly distinguished since 1958 This organism is aerobic catalase positive and consistently gram positive forming filaments often with elongated swellings and sometimes showing branching A relationship to *L buccalis* and to *Nocardia* was suggested by Davis and Baird Parker (1959b) on the basis of the finding of DAP in the cell wall Common antigens have been found by complement fixation with *Nocardia Mycobacterium Corynebacterium* and *Actinomyces* but not with *Lactobacillus* (Schmidt and Richardson 1962) Sibal and co workers (1962) using acid soluble antigens in tube precipitin and gel diffusion studies found little or no cross reaction with *L buccalis Actinomyces* spp *Nocardia asteroides* or *Lactobacillus* spp

Mycobacterium There are suggestions that several species of mycobacteria other than true tubercle bacilli occur in man frequently enough in the absence of symptoms to be thought of as indigenous These include *M smegmatis* present in external genital secretions according to early literature and other members of the so-called atypical or unclassified mycobacteria (see Chap 21)

Clostridium *C perfringens* (type A) is found commonly in human feces *C tetani* common in animal feces has been recovered only irregularly in man More typical soil clostridia have also been found occasionally in man but as with members of the genus *Bacillus* under similar circumstances may probably be dismissed as transients

GRAM NEGATIVE AEROBIC BACILLI
(See also Chap 25)

Enterobacteria The easily grown aerobic gram negative rods although outnumbered in the intestinal tract by anaerobes of other genera are the best known of indigenous bacteria

Moraxella and Mima Certain gram negative aerobic bacilli on which a large literature has been accumulating are distinguished by neisserialike characters coccal as well as bacillary (and filamentous) forms a tendency to gram variability intracellular occurrence pathologically in spinal fluid or genitourinary exudates and a gradient of properties ranging from oxidase positivity and penicillin sensitivity to the reverse The former are also generally glucose nonfermenters whereas the oxidase negative penicillin resistant forms tend to be aerobically glycolytic Variation from apparently obligate parasitism to saprophytism is correlated with decreasingly exact ing nutritive requirements The principal features of the 3 species defined tentatively pending more adequate taxonomic study of the group are given in Table 1

Moraxella lacunata (the Morax Axenfeld bacillus) here includes *M duplex* and its variants *Mima polymorpha* is considered to be a transitional group some strains of which are oxidase and penicillin positive similar strains giving negative reactions constitute the *Moraxella lwoffi* of French workers *Mima vaginicola* the glycolytic group appears under a variety of names including the genera *Herellea Achromobacter* and *Acinetobacter* as well as *Moraxella* and the spe

appearance of tufts of flagella in light microscopy has given rise to the apparently erroneous practice of considering this organism to be a protozoan (*Selenomonas sputigena*) *S sputigenum* appears to be an unusually fastidious anaerobe difficult to cultivate on agar surfaces but growing well in thioglycollate broth under petrolatum

Vibrio sputorum is a small usually curved polar flagellated gram negative organism with pointed ends Its motility is rapid darting often in an elliptical course and characterized by stops sometimes with reversal of direction It grows anaerobically on enriched agar surfaces or as stab cultures in semi solid agar but poorly in broth It is non saccharolytic

Butyrivibrio fibrosolvens a small motile gram negative anaerobe originally isolated from the rumen was recovered from high dilutions of human feces in 2 of 7 trials by Brown and Moore (1960) who also provided evidence that the organism may participate in fermentation in the human intestine producing butyric and other fatty acids

SPIROCHETES
(See Chap 24)

These flexible highly motile nonflagellated microorganisms include anaerobic forms indigenous to mucous membranes Usually sparse in the absence of inflammatory disease they may occur in enormous numbers under many pathologic conditions Since gingivitis commonly mild or localized is seldom entirely absent spirochetes can be demonstrated nearly universally best at the bottom of the gingival crevice or pocket

The amphibiotic spirochetes have been cultivated principally by variations of the Noguchi method in the depths of enriched agar media Means for isolation from surface colonies have been proposed in recent years (Socransky *et al* 1959 Hardy *et al* 1963) The validity of the current classification of the indigenous spirochetes is doubtful Cultures derived from different sites such as mouth intestine and genitalia, have been arbitrarily given different names The evidence suggests that both of the anaerobic parasitic genera *Treponema* and *Borrelia* are represented among indigenous forms but until more reliable information is at hand only a single species of each need be named

Treponema dentium (*T microdentium* etc) is a pallidumlike spirochete tightly wound single contoured capable of active motility with rapid rotation darting progression and flexing movements suggesting greater rigidity than do the typically soft undulations of *T pallidum* Listgarten *et al* (1963) found in electron micrographs an axial filament made of 2 fibrils (*T pallidum* has 3) approximately 150 Å in diameter lying between an external envelope and the protoplast The organisms are usually actively proteolytic but many cultures do not ferment carbohydrates They appear to be very fastidious both in their nutritive requirements (see Pillot 1962) and in their need for anaerobiosis Their pathogenic proclivities remain poorly defined These forms rather than *Borrelia* spp seem to be most consistently associated with fusospirochetal disease but most attempts to demonstrate experimental pathogenicity have yielded at most nontransmissible disease (see Hampp and Mergenhagen 1961 1963)

Borrelia refringens These loosely wound indigenous spirochetes have been defined only provisionally The name assigned by Schaudinn and Hoffmann in 1905 to a genital form (which they distinguished from the spirochete of syphilis) has priority Forms with single contours (called *B vincentii* in the mouth) and double contours (*B buccale*) are probably variants of one species Many other synonyms have been used The pitch (crest to-crest distance) of *B refringens* is usually more than twice that of *T dentium* (approximately 2 5 to 4 microns as compared with 1 2 microns) The coils are regular when the spirochetes are viable and active Swain (1955) gave the following measurements from electron micrographs thickness 0 23 to 0 64 micron average 0 42 micron 8 to 11 axial fibrils 200 Å wound helically around the helical protoplast In active motion these spirochetes spin rapidly and seem to stretch their coils almost taut as they move in straight lines they may stop intermittently relax their winding and reverse their direction Slower movements are less characteristic These spirochetes stain more easily with aniline

bic rod which has been found together with *Actinomyces israelii* in actinomycosis as has the equally obscure so called *Actinobacillus actinomycetemcomitans* Both organisms have also been found under normal conditions The two forms appear to be distinct (see King and Tatum 1963)

Gram negative Anaerobic Bacilli and Spirilla
(See also Chap 32)

Bacteroides, Fusobacterium A large and varied group of amphibiotic obligately anaerobic gram negative rods some of which are fusiforms or filamentous or highly pleomorphic have been classified under many names, both generic and specific but are grouped here pending further study into two related genera and a restricted range of species The bacteroides are probably the most numerous organisms in adult human feces (see Zubrzycki and Spaulding 1962) On the other hand Haenel (1961) made no mention of gram negative anaerobes in reporting on 50 analyses of feces of 21 healthy adults According to this worker the predominant organism in feces (10^9 to 10^{10} per Gm) is a facultatively anaerobic lactobacillus They are usually nonfusiform but range in morphology from regular rods to filaments and highly pleomorphic forms All but one species (*B serpens* one of the least understood of this neglected group) are nonmotile The fusobacteria include one motile and one nonmotile species

Bacteroides fragilis probably the commonest species in feces is nonpleomorphic saccharolytic (pH in glucose 4 6 to 5 4) and penicillin resistant It usually requires enriched media *B funduliforms* (*B* [*Sphaerophorus*] *necrophorus*) the highly pleomorphic form most commonly implicated in disease is a moderately weak fermenter (pH 5 6 to 6 5) grows in simple media after isolation and reacts variably to penicillin It produces H S and NH_3 and a predominantly butyric fermentation *B nigrescens* (*B melaninogenicus*) is a distinctive species or group that forms a black pigment (a hematin derivative) from hemoglobin after 3 to 5 days growth These pigment formers have been intensively studied in recent years under the hypothesis that they may be significant contributors to certain mixed anaerobic infections as noted below Sawyer *et al* (1962) found that all of 31 strains actively hydrolyzed collagen and gelatin and produced H S All required or were stimulated by hemin some strains required menadione or related naphthoquinones Wide variation in fermentative activity was observed *B nigrescens* is sensitive to penicillin and to other antibiotics

Bacteroides (*Dialister*) *pneumosintes* is the name assigned to a small gram negative anaerobic bacillus first isolated from the nasopharynx during the 1920 s and said to be filterable through Berkefeld V and N filters A single strain obtained from Prevot was studied by Sonnenwirth (1960) and more recently by Chien-Ching Chen and Cleverdon (1963) Except for its small size the organism seems to be related to *B fragilis* The latter workers established its maximum diameter as 0 4 to 0 6 micron by Millipore filtration

Fusobacterium fusiforme is the typical small nonmotile fusiform bacillus found on normal mucous membranes and in fusospirochetal disease Morphologically it resembles *B funduliformis* but is more clearly spindle shaped and less pleomorphic It differs also in being less acidogenic in glucose (final pH not less than 6 0) in requiring enriched media and in being uniformly sensitive to penicillin This organism has been confused with *Leptotrichia buccalis* from which it differs in morphology and metabolism Confusion with spirilla (e g *S sputigenum* see below) and spirochetes was formerly common *F fusiforme* is easily and clearly separable from both

Fusobacterium girans is a slowly motile nonflagellated gram negative anaerobic fusiform found in the mouth

Anaerobic Spirilla and Vibrios A group of curved or helical flagellated anaerobic bacteria is found in areas of abundant mucous membrane flora and is associated especially with fusospirochetal overgrowths Among these can be distinguished *Spirillum sputigenum* a variable comma shaped S shaped or helical form showing active motility This organism is typically gram negative but may appear to be weakly positive Its flagella are peritrichous but the

E h h l						±		4 2		++	67 99	100		+	+ 6
I t med tes			0 1–0 4				±	31			28–5	+		+	
Kl bsi ll gen			+	0 1		±	+	52		++	19–48	33–68			
I teu m l lis th							+	+			48	5–53		+	+
pp			0 2 1	0 4								–6/Gm			
P eud m rug a			0 1 3				±				+ 0 2 1	3 11 +		+ ±	
Al l g n f l			1 1 1 6	±				±				±			
V bro l l g se													±	±	
M az ll l cu t				±	+	±						±			+
M m p lym ph				±		±						±		5	+
M g n l				±		±									+
H mophil nfl				0 4 2 5	12	43 90	3 97	5 100							
H p fl en						5	20	25							
H m lyti h m ph l						+	77	+							
H gypt				+										±	+
H g n l															
E A h g m g t b lli b l pi ill d pi h t															
PPI O t															
B t d f g l						+	+	+	+	+		100?	+		±
th pp												7 10/Gm			
B n g en								+	+ 6/mg			+	+		
Fu b t m f f							+	+	14 88 3 5/ml	+ 4/mg		+	+		
F g									+			+			±
Sp rill m p t g m						+		±	+	+		+?	+		+
V b put rum						+	+	±	+	+					
T pon m d t m d B l f g							+			60–88 6/mg	+	18	8	+		
PPLO t							+		+				+	+	±	+
F F l C nd d alb	±	±	+				6 8		6 49 0 5/ l		+	+	14 31 0 4/Gm	+	±	28 46
Oth d d s	1 15	+	+				3 10 ±		1 4 +				1 12 +	+	±	4–6 +
T rul p gl b at																
P ty p rum al	100															
P b ul	++															
D rm t phytes		2 41														

±0 ra ± i g l o u ta (m y be ly p th l gl) + mm ++ pr mi t rv ys V l es g n w th u its (g 3–6/ml) = ra g of nc nt ti p ed l g10 6 = >5 × 10 <6 × 10
B ldf l es (g 31 59) = ra g f i ld n i p t, nd d i d ff rent

† + i b n m mm n i hool hild n th n in dults
‡ P d mi t fi t d y dec i g d i g first m nth
§ A ted w th n l rr g
¶ l t f t n l ted
I f t d h ld h ghest h p tal nursery i f ts
ᵃ Childre
I wt
M e mm n b l w g 0
M mm h l h ld n
ᶠ A i t d with p es th t
H m lytic L fl ld g p n t gl e
(F m Ros b y [1962] M G w Hill B k C)

ʰ G p B C F and G n A
Ch ld n t g p A
ʲ M mm n hild
E pec i y n d ntal ies
B ttle-f d f nt
ᵐ B ast-f d i f ts
Ch ld
Aft th se nd w k
C t ll t ul
E p lly lp d l f ld l th sk a

TABLE 2 MICROORGANISMS COMMONLY FOUND ON HEALTHY HUMAN BODY SURFACES*

	Skin				Upper Respiratory Tract			Mouth			Lower Intestine			Genitourinary Tract		
												Feces				
Species or Group	Central	Feet	External Auditory Canal	Conjunctiva	Nasal Passages	Nasopharynx	Oropharynx tonsil	Predentulous	Saliva Tooth Surface	Gingival Crevice	Upper Levels	*Infant*	Adult	External Genitals	Anterior Urethra	Vagina
A Gram-positive cocci																
Coagulase-negative staphylococci	88–100 2 6/cm		27–100	37 94	90	++	±†	+	75–100 1 4/ml			11–59‡	+ 4 Gm	+	++	34–78
Coagulase-positive staphylococci	5–24§		12–20	0–30	22–85 9–100	++	36–42		+ (16–35)¶		+	10–93	++§		±	5–15
Anaerobic micrococci	±						+		+		+					+
Streptococci, nonhemolytic and undifferentiated and α and γ streptococci	±0		0 2–23	0 9–1	±	24 99	100	+	100 6 8/ml	100 6/mg	+	14 32	+	+	±	10–21 47–50
St. hominis (salivarius)						+	++	100	100 /ml	++	+	0–6	+			
Enterococci or group D streptococci							+	+	4 22[d]		+	87 6 9/Gm	100 3 8/Gm	+	-10	+
St. pyogenes (usually group A unless noted)	0 4			0 3 2 5	0 1 5	0–9	5 66		12–68[f] 3–6/ml[f]			0 7–19	16[h]			4 4–10[i]
Anaerobic streptococci							+	+	++	++ 6/mg			+	+		12–59
D. pneumoniae			+	0–5	0–17	0 50	8 72	+	26							±
B Gram-negative cocci *Neisseria catarrhalis* and other spp				2 3	12	10–97	98		95–100 5 7/ml	+					±	+
N. meningitidis *Veillonella alcalescens*						+ +	+	+	100 6 8/ml	+						+
C Gram-positive bacilli Lactobacilli								+	95[k] 0–6/ml		+		60[a] ~7/Gm			49–73
Aerobic corynebacteria	53 6/cm		86	3 83	++	+	+		59			10–21	6	+	+	44–74
C. acnes	45 100 6/cm				+			±								
Mycobacteria	+				±	±	±									
Cl. perfringens, other spp													+	+	±	
Cl. tetani									±		++	13 19	25–35 1–35			
A. bifidus								+	+			15–60 90[m] 11/Cm[m]				6 72
A. israelii							+		+	+						
Leptotrichia buccalis									++ 0–3/ml	++						±
L. dentium									+							
D Anaerobic gram-negative bacilli Undifferentiated forms			4–8	2 1		21 3	+	+	65 0 3/ml			86–100 7 9/Gm	100 8 8/Cm	+	+	17–34 3 12

of averages (2) the colony count per unit of surface volume or weight given as a range in rounded $\log s_{10}$ and (3) where the preceding data are not available a rougher measure of occurrence or prominence in terms of ± 1+ or 2+ Spaces left blank usually indicate absence of information

In body sites not given in the table the prevailing condition in health is presumably either microbiologic sterility as in blood and tissues or a sparse biota approaching extinction or in passage as in larynx trachea bronchi accessory nasal sinuses esophagus stomach upper portions of the intestinal tract upper urinary and posterior genital tracts and subjacent structures The healthy conjunctiva and anterior urethra also have a scanty flora and the nasal and the vaginal mucous membranes are relatively lightly populated

Microbic colonization begins at birth the first settlers being transferred from the maternal vagina and perineal area to the infant skin mouth nose and oropharynx The characteristic biotas of these and other areas develop later and probably are derived and selected from various environmental sources The character of the amphibiotic relationship and the relative uniformity of the flora in widely scattered persons suggest an origin principally in other human beings

It may be useful to subdivide the indigenous microbiota of man into 3 groups In the first of these (the lower intestine) extraneous pabulum is present in massive amounts and tends to favor the overgrowth of saprophytes (although true amphibionts e g bacteroides still predominate) In the second—the mouth the oropharynx and the vulva—pabulum is relatively abundant enriched either by extraneous material (from ingested food) or accumulated cell debris or both crypts or recesses particularly favor anaerobes The third—the skin—differs in its lower moisture and high lipid contents which tend to select a somewhat different biota It is worth emphasizing that in general strict anaerobes tend to outnumber facultative forms and aerobes usually by a factor of 10 to 100 or more This is true even in skin and in the highly ventilated nasal passages

SIGNIFICANCE OF THE INDIGENOUS BIOTA

Under the usual conditions of exposure man s skin and the mucous membrane lined orifices leading inward directly from it rapidly become populated with a varying but nevertheless characteristic range of microorganisms These are assumed to be in continual interaction with the host and with other species within the biota itself leading to fluctuations in the biota both qualitative and quantitative and to more or less pronounced effects on the host These effects range from beneficial through inapparent to detrimental (See also Dubos *et al* 1963)

NONPATHOGENIC ACTIVITIES

The discernible nonpathogenic effects of the normal flora on the host include (1) nutritional effects resulting from microbial synthesis (2) various interactions between microbiota and host (3) protective effects of latent infection (4) direct and indirect consequences of interactions between the microbes themselves including antibiotic effects and (5) other phenomena that can be recognized either directly or by inference from experimental studies of germ free animals

Germ free animal studies merit more extended discussion than space permits (see Rosebury 1962 and the review by Mickelsen 1962)

Several species of vertebrates have been bred through successive generations in a germ free state Thus the biota is not essential for their continued existence Experiments in which germ free animals have been contaminated deliberately or accidentally have begun to provide much basic information on the effects of the microbiota which will be considered under the following heads

The Indigenous Biota in Host Nutrition Despite the fact that the microbiota is not essential to host life considerable evidence indicates that the normal intestinal flora contributes to host nutrition Some members of the biota especially the nutritionally nonfastidious enterobacteria, synthesize vitamins

dyes, e g, crystal violet than the tightly wound forms they are typically distorted in routine dried films Cultivation of the loosely wound forms has been reported less frequently than of *T dentium*, especially from the mouth Detailed methods that might permit cultivation of *B refringens* apart from the other forms have not been reported Statements as to its nutritional and metabolic characteristics are to be regarded with caution It has been suggested that these loosely wound spirochetes share the elusive pathogenic attributes of the tightly wound forms (Hampp and Mergenhagen 1961)

Fungi
(See also Chap 37)

Nearly all fungi found in man are saprophytes widely distributed in the environment Therefore the separation of indigenous species is more than ordinarily difficult Exceptions appear among yeastlike fungi The association of *Pityrosporum* species (*P ovale* and *P orbiculare*) with the skin may be due to its requirement for fatty acids or glycerol *Torulopsis glabrata* also occurs on the skin and the mucous membranes it is said to require biotin thiamin niacin and pyridoxin for growth at 37 C The dimorphic yeastlike form *Candida albicans* although capable of growing saprophytically seems to be limited in its distribution to parasitic sites Other species of *Candida* namely the closely related *C stellatoidea* and *C tropicalis* may be true amphibionts while still others—*C parapsilosis C krusei C guilliermondi* and *C pseudotropicalis* although saprophytic appear on mucous membranes often enough to be thought of as indigenous *T glabrata* and the *Candida* species can cause experimental disease in mice and have also been implicated in human disease (see e g Hurley and Winner 1962) *C albicans* is evidently the most pathogenic of these forms but even this species causes disease only when the infecting dose is large or when certain abnormal conditions exist for example when the experimental animal has been pretreated with cortisone or x-radiation Prolonged treatment with broad spectrum antibiotics often leads to the appearance of candidiasis in man and also facilitates the establishment of *C albicans* in the mouse intestine (see Sheldon and Bauer 1962)

Certain of the dermatophytes of tinea pedis namely *Trichophyton rubrum T mentagrophytes* and *Epidermophyton floccosum* may be included among amphibionts because of their widespread occurrence on healthy skin of the feet and also because certain aspects of both the naturally occurring disease and of its experimental reproduction in man suggest endogenous infection (see Barlow *et al*, 1961, but also Ajello 1962)

Although *protozoa* are outside the scope of this book, it should be noted that members of this group are indigenous to the human mouth intestine and vagina (see Rosebury, 1962)

Pleuropneumonialike and Related Forms
(See also Chap 35)

The interrelationship between so-called PPLO (pleuropneumonialike organisms), L phase variants of bacteria and bacteria from which the cell wall has been denuded experimentally partially or completely (spheroplasts and protoplasts) is still unsettled For this reason it is inadvisable to apply separate generic and specific names to these forms (Rosebury, 1962) In the present context it may be noted that both PPLO which are not known to revert to bacteria and L forms which are have been recovered from oral pharyngeal anorectal and genitourinary mucous membranes both in health and in disease They are recovered more commonly under pathologic conditions and have been held responsible for disease especially nonspecific urethritis but experimental pathogenicity generally has not been demonstrable

DISTRIBUTION OF THE INDIGENOUS MICROBIOTA

The distribution of species or groups of bacteria and fungi in various sites are summarized in Table 2 Data are given in the table in 3 ways (1) the incidence as a percentage of subjects studied given as a range

of averages (2) the colony count per unit of surface volume or weight, given as a range in rounded $\log_{10}$ and (3) where the preceding data are not available a rougher measure of occurrence or prominence in terms of ± 1+ or 2+ Spaces left blank usually indicate absence of information

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in excess of their own requirements, and there is evidence that such microbes contribute to host nutrition by supplying significant amounts of biotin, vitamin K, pantothenic acid pyridoxine and vitamin B_{12} In germ free rats there is striking evidence of a need for dietary vitamin K which is not required by conventional animals (Gustafsson 1959)

Host Biota Interactions Other Than Disease Whenever a given microbic species is found regularly and in appreciable numbers in an indigenous locus it may be assumed that its metabolic activity directly or indirectly influences the host

Observations on germ free animals indicate that the indigenous flora may exert on the host beneficial effects not directly related to nutritional supplementation For example in the absence of an intestinal flora guinea pigs and other animals develop an unusual anomaly cecal distension (see Mickelsen 1962) Skelly *et al* (1962) found that enlargement of the caecum in germ free mice could be corrected by feeding anaerobes isolated from the ceca of normal mice a clostridium or a mixture of two strains of bacteroides Germ free animals are also abnormal in many biochemical activities as well as in their greater susceptibility to infection (references in Rosebury 1962) Phillips and Wolfe (1961) observed spontaneous pneumonic disease of presumed viral origin in germ free guinea pigs which could be cured by feeding autoclaved cecal contents of conventional animals (See also Lev 1961)

Latent Infection Antibody Formation Antibodies have been found in healthy human serum or other body fluids to the following microorganisms listed above as indigenous coagulase positive staphylococci nonhemolytic streptococci lactobacilli actinomyces of the israelii group *E coli* and other enterobacteria *P aeruginosa H influenzae B funduliformis* fusobacteria indigenous spirochetes and *Candida albicans* and other members of this genus The significance of these antibodies is not clear but it is probable that they indicate past invasion and presumably subclinical disease Findings in germ free animals make it likely that such antibodies help to protect the host against further invasion by the microbic species concerned The question has been explored especially in germ free rats in which lymphatic tissue was found to be poorly developed (Thorbecke *et al* 1957) There was an associated marked reduction in serum globulin and in antibody activity Grabar *et al* (1961) found that only small amounts of γ globulin were detectable in germ free rat serum by direct analysis although all major antigenic components in conventional rat serum could be demonstrated by immuno-electrophoresis of anti germ free rat immune rabbit serum However germ free mice have normal levels of antibody to endotoxins of gram negative bacilli presumably in response to antigens present in food and water (Landy *et al* 1962) In some instances germ free animals are susceptible to disease induced by indigenous microorganisms for example *Sarcina lutea* and *Bacillus subtilis* (Taylor 1959) but in other cases they have been contaminated with normal feces without developing disease (Gordon 1959)

Interactions of Indigenous Micro organisms Both cooperative (synergistic syntrophic) and competitive interactions occur among indigenous microbic species and between these and either saprophytic or pathogenic intruders Our knowledge of such interactions is generally meager Their study presents formidable difficulties inherent in the vagaries of population mixtures comprising 2 or more species (see Parker and Snyder 1961 Jameson 1962)

Cooperative interactions (other than genetic exchange) include (1) satellitism the production by one species of a condition (e g a low oxidation reduction potential) or a substance (e g a growth factor) which permits contiguous growth of another species and (2) enzyme sharing e g permitting growth of two species in media inadequate for either alone or yielding a joint product not found in the separate pure cultures For examples see Rosebury (1962) and also Freter (1962)

Competitive interactions probably depend on either or both of two phenomena (1) competition for nutrients and (2) the production by one species of conditions (e g a low pH) or substances (antibiotics) adverse for others Antibiotic effects have been described for the following indigenous species

(1) *Staphylococcus aureus* and other staphylococci against corynebacteria including *C diphtheriae* *Bacillus* spp streptococci including *S faecalis* mycobacteria clostridia other staphylococci and other species (2) Nonhemolytic streptococci especially viridans forms (and pneumococci) acting against a wide range of test species including staphylococci cornynebacteria neisseria and many others among them β streptococci Some of these streptococcal effects are known to be due to H O and some including the α β effect are mutual inhibitions effected by the more against the less abundant species (3) Lactobacilli (in addition to their effects attributable to low pH) inhibit staphylococci enterobacteria and other organisms (4) *Pseudomonas aeruginosa* produces a range of antibiotic substances against *B anthracis* *V cholerae* staphylococci streptococci enteric pathogens and others as well as against *Candida albicans* and other fungi This species also produces *pyocines* analogous to *colicines* (see below) (5) Enterobacteria especially *E coli* have been found to be inhibitory in vitro for *Candida albicans* and *C krusei* and for several bacterial species other than enterobacteria themselves but such antibacterial effects have seldom been striking or easily demonstrable The name *colicines* has been applied to an apparently diverse group of substances produced by certain enterobacteria especially certain strains of *E coli* which are inhibitory in vitro for other enterobacteria frequently for other strains of the producing species often also for *Sh sonnei* Goebel and his co workers (see Goebel and Amano 1959 Hutton and Goebel 1962) have found two colicines (K and V) to be associated with the O antigens of the effector strains The strain specificity of colicines has led to their use in typing enteropathogenic *E coli* and other enterobacteria

PATHOGENIC EFFECTS OF THE INDIGENOUS BIOTA

Endogenous Infective Diseases Some General Principles

The subject to be surveyed here is so varied that no attempt can be made to cover it completely Both the idea that indigenous micro-organisms may participate in disease in the presence of predisposing conditions and the term applied to such disease endogenous infections seem to have originated with Escherich in 1889 Although the idea has found support in relation to particular diseases and seems never to have been abandoned it has had a discontinuous history The apparent difficulty that such processes violate an unstated corollary of Koch s first postulate—the alleged causative agents being found in health as well as in disease (the postulates are reviewed in Editorial 1961) and difficulty with the third postulate—isolated pure cultures are nearly always nonpathogenic under customary test conditions—may have contributed to this neglect Today these obstacles no longer block the road the carrier state and silent infections negate as a fallacy the assumed corollary to the first postulate fulfillment of the third postulate has been accomplished often enough to establish the principle

Before presenting a few examples of endogenous infective diseases it may be useful to outline certain features common to the whole group It has been pointed out already that diseases associated with indigenous microorganisms cannot be sharply distinguished from exogenous infections in which latency or its equivalent is a conspicuous feature Indeed it may be noted that the very word endogenous is used only for convenience The group differs from endogenous reinfection in tuberculosis for example principally in that *M tuberculosis* is easily recognizable as a pathogen

The common features of endogenous infective diseases may be listed as follows (1) Microorganisms found in the indigenous biota are indispensable agents in the pathogenesis of endogenous infective diseases (2) The microbial agents of endogenous infective diseases occur in the indigenous biota in health but in disease they are either significantly increased in concentration in or near their usual sites or are found proliferating in an unusual site—in the tissues (3) The bacterial agents of endogenous infective diseases are characterized by low intrinsic pathogenicity (4) A state equivalent to latency in exogenous infections is characteristic of endogenous infective diseases which there

fore have no definable incubation period (5) *Endogenous bacterial diseases are not com*municable within the accepted limits of meaning of this term (6) Immunity, in the sense of specific protection against recurrence of a given endogenous infective disease is not recognized clinically rather the diseases have a marked tendency either to recur or to progress slowly over a period of many years (7) In endogenous infective diseases, causes or determinants other than the *activity of indigenous microbic agents are as* indispensable to pathogenesis as are the agents themselves Another feature of this group of diseases frequent but apparently not universal is that they are likely to be nonspecific with respect both to their microbic agents and to other determinants of *pathogenesis It is usually futile* to search for single causes of diseases in this group, or otherwise to attempt to deal with them by analogy with disease outside the group

SUBACUTE BACTERIAL ENDOCARDITIS

Subacute bacterial endocarditis (s b e) is a characteristic endogenous infection whose pathogenesis is rather well understood and comparatively simple Implantation on a previously damaged heart valve of nonhemolytic streptococci or other microorganisms follows an initial transitory bacteremia induced by surgical or comparable manipulation The organisms proliferate in and under cover of fibrin platelet thrombi which protect them from phagocytosis and other blood clearing mechanisms forming vegetations which lead to persistent bacteremia and in untreated cases to death by occlusion of the affected valve, other vascular accidents or serious embolism The microbic agents concerned are usually nonpathogens , pathogenesis seems to require of them only an ability to proliferate under conditions provided by the host

There seems to have been no marked reduction in the incidence of s b e since the advent of the antibiotic era (see e g Pankey, 1961), and although modern treatment has reduced mortality markedly—formerly it approximated 100 per cent—deaths continue to be common seldom fewer than 20 per cent of cases even in small favorable groups (e g Hamburger *et al*, 1961), and as high as 72 per cent in a group of older patients (Cummings *et al* 1960)

In s b e, indigenous viridans streptococci continue to be the most common organisms enterococci are also found in a significant minority of instances (see Toh and Ball 1960 Koenig and Kaye, 1961 Tompsett and Pizette 1962) Groupable streptococci other *than A D or N hemolytic or not have also* been found In addition many diverse indigenous species appear including uncommonly or rarely, such otherwise benign species as lactobacilli or veillonellae Mixed infections are found but not frequently A considerable proportion of cultures remain sterile perhaps in part because routine methods do not disclose them—as would be true of fastidiously anaerobic amphibionts (for recent reviews see Jackson and Allison 1961 Quinn and Colville 1961, Vogler *et al*, 1962a b)

Pre-existing cardiac damage most commonly affecting the mitral valve is an important antecedent of s b e In approximately 75 to 92 per cent of instances in which such damage is recognized it is rheumatic in origin in the remainder it may be arteriosclerotic congenital or syphilitic In many instances of rheumatic valvular injury the patient is unaware of the defect before the onset of s b e

The streptococci most frequently implicated in this disease and many of the other species recovered from blood cultures are nonpathogenic for laboratory animals by the usual routes of inoculation However progressive disease closely similar to s b e in man has been produced experimentally in animals by a variety of means including cardiac damage induced before intravenous inoculation of nonhemolytic streptococci Highman *et al* (1952) showed that cardiac damage could be induced in rats exposed intermittently for prolonged periods in a low pressure chamber to simulated altitudes of 25 000 feet Some of these animals developed spontaneous bacterial implants Repeated intravenous injection of *S mitis* or a single large dose of *S faecalis* yielded bac

terial endocarditis in a majority of the animals (See also Altland *et al* 1959 Walker and Hamburger 1959)

The bacteremia which leads to s b e is of diverse origin With indigenous microorganisms the mechanism may include trauma manipulation or massage of a mucous or skin surface Tonsillectomy and dental operations have been particularly incriminated especially in cases involving viridans streptococci However evidence for the association is often lacking It is recognized that manipulation of other loci with a normal flora (e g the genitourinary tract see Lein and Stander 1959 Koenig and Kaye 1961) may be responsible Dental interest in this problem is reflected in a considerable literature in which in addition to tooth extraction and other oral surgery (e g Schirger *et al* 1960) transient bacteremias have been induced especially by gingival and periodontal manipulation (Vargas *et al* 1959 Gutverg and Haberman 1962 Korn and Schaffer 1962) On the other hand cavity preparation pulpotomy (Beechen *et al* 1956) and root canal operations (Bender *et al* 1960) have been found not to induce bacteremia Bender and his co workers recommended that such treatment be chosen in preference to extraction in persons with valvular disease when this is feasible The occasional alternative suggestion that tooth extraction be performed prophylactically in susceptibles seems to be of doubtful validity Massaro and Katz (1960) recorded an instance in which viridans streptococcal s b e occurred in an edentulous man and mentioned 2 earlier cases

Specific therapy of s b e caused by penicillin sensitive streptococci (briefly reviewed by Hamburger *et al* 1961) continues to favor a combination of penicillin with streptomycin This combination also has a good record in enterococcal endocarditis even when in vitro sensitivity tests have not been favorable (Koenig and Kaye 1961 Tompsett and Pizette 1962) Control measures usually emphasize the administration of antibiotics before during and after surgery or other potentially precipitating manipulations in patients with known or suspected cardiac damage Several studies of this procedure agree that it reduces but does not abolish the incidence of bacteremia (e g Shirger *et al* 1960 Gutverg and Haberman 1962)

ACTINOMYCOSIS

A subacute or chronic usually progressive disease of orofacial thoracic or abdominal tissues actinomycosis produced by *A israelii* alone or perhaps in combination with other microorganisms is another clear example of endogenous infection In this instance however while host factors in pathogenesis seem to be essential to permit or enable the occasional invasion by a surface parasite the nature of these factors is poorly understood Actinomycosis occurs in man cattle and other animals It is characterized in man by the development of indurated granulating swellings chiefly in connective tissue by suppuration usually of limited extent and by the presence of *A israelii* in the pus or lesions The disease develops over periods ranging from a few weeks to a year or more and may spread widely by contiguity sometimes pointing toward the skin and forming fistulae that tend to heal and re form elsewhere rarely pointing toward mucous or serous membranes The organism may be disseminated through the blood or in the lungs through the bronchi The lymphatic system is only rarely involved Bone lesions are uncommon in man

Actinomycosis is generally thought to occur in the cervicofacial region somewhat more frequently than in all others combined Such cases are seen most frequently in dental or oral surgical clinics Fisher and Harvey (1956) found that of 90 cases treated over a 25 year period 55 per cent involved the abdominal wall or viscera 23 per cent were thoracic 13 per cent were cervicofacial and 9 per cent appeared in other areas The commonest *cervicofacial* lesions are seen on the cheek or submaxillary skin as indurated or edematous swellings often bluish or reddish in color with a tendency to form a series of irregular folds separated by furrows the healing area forming scars as new lesions develop *Thoracic* actinomycosis is found mainly in the lungs with the formation of abscesses and cavities which are usually small Extensive abscesses may be found in the bronchi and

their rupture may lead to dissemination of the infection by way of the bronchial tree Actinomycotic pleurisy and empyema have been observed as has involvement of the heart and the pericardium *Abdominal* actinomycosis may be found in any organ but is most common in the region of cecum and appendix From here the lesion may extend with suppurative foci and the formation of fibrous adhesions to the abdominal wall where skin lesions may appear similar to those of cervicofacial actinomycosis Or the lesion may remain circumscribed forming a fibromalike mass The liver is commonly attacked and lesions of the genital tract are relatively frequent The stomach the small intestine and the kidney are seldom affected Rarely actinomycosis may be found in the anorectal area or the testis

The microscopic appearance of the lesions of actinomycosis varies from that of an acute abscess with an abundance of polymorphonuclear cells to the more chronic lesion in which proliferating connective tissue is the most conspicuous feature Commonly the picture includes necrosis with an abundance of leukocytes surrounded in turn by granulation tissue and a profuse formation of dense fibrous tissue It is thus not characteristic unless *sulfur granules* are present These are frequently lacking in either tissue or pus and when present particularly in lesions in man may not show typical clubs Club bearing granules may be found in several distinct diseases distinguishable from those of true actinomycosis in gram stained but not in routine hematoxylin eosin sections Details of the typical granule are seen best under magnifications of 400 diameters or more It may be roughly circular or irregular in outline or may consist of several colonies of different size and shape that have coalesced The granule is composed of a dense reticulum of fibrils which stain violet by Gram s method but may otherwise stain irregularly Around the periphery the ends of individual filaments may project, with or without radially arranged hyaline clubs The clubs when present, take the eosin stain and are several times larger than the filaments whose ends they enclose Pine and Overman (1963) have found sulfur granules (of *A bovis* from cattle) to have essentially the same composition as organisms grown in vitro except that the sulfur granule contained about 50 per cent calcium phosphate

The epidemiology of actinomycosis is that of an endogenous infection as was clearly shown by Wolff and Israel in 1891 and by Wright in 1905 Nevertheless the pathogenesis of actinomycosis is incompletely known In the hands of earlier workers attempts to demonstrate pathogenicity of *A israelii* for common laboratory animals by the usual routes were usually unsuccessful or yielded only localized lesions

Repeated passage, trauma during inoculation or admixture with other bacteria were generally ineffectual However progressive and fatal experimental actinomycosis with typical granules and other pathologic signs has been produced—although again without regularity—by repeated inoculation at intervals that might have permitted development of allergy to the organism but allergy could not be demonstrated convincingly Meyer and Verges (1950) and Geister and Meyer (1951) have reported the consistent production of actinomycosis in mice by injecting pure cultures in association with gastric mucin Hazen *et al* (1952) produced typical actinomycosis in 21 of 28 young male hamsters with any of 8 strains of *A israelii* Gale and Waldron (1955) found that actinomycotic lesions with typical granules usually without clubs could be produced in mice with any of 7 strains of *A israelii* but the disease developed during the first week after inoculation and then tended to recede Guinea pigs inoculated with a single strain responded similarly These results may help to explain the failure of other workers who depended on gross illness or death as a sign of experimental infection Strains of *A israelii* isolated from mucous membranes have been found as capable of inducing lesions as strains obtained from the natural disease The experimental findings as a whole leave the pathogenesis of the progressive disease in man uncertain The comparative rarity of actinomycosis suggests that autoinoculation resulting from minor trauma perhaps even when repeated can be no more than a contributory incident More severe traumatic events have often been associated with actinomycosis e g a tooth extraction or other

injury to mouth or throat human bite or knuckle injuries from a blow to the teeth or aspiration of an extracted tooth or tooth fragment into the lungs *A israelii* has been found in salivary calculus and detached masses of tartar may be involved in comparable traumatic accidents Actinomycotic pus from closed lesions frequently perhaps invariably contains other organisms in addition to *A israelii* the suggestion has been made that a mixed infection may be important in establishing the disease

Although the finding of microscopically typical sulfur granules is presumptively diagnostic of actinomycosis in man the diagnosis cannot be considered as established unless *A israelii* is isolated from the lesions For this purpose plates should be serially streaked on brain heart medium containing 2 per cent agar or on blood agar and incubated anaerobically with 5 per cent CO for 4 to 6 days at 37 The opaque white heaped up colonies of *A israelii* particularly the rough colonies can be identified easily even in the presence of abundant contaminating growth but smooth colonies may be indistinguishable from those of diphtheroids Isolation may be made into tubes containing a 10 cm column of glucose infusion broth in which the organism typically grows after aerobic incubation as a crumblike or tiny cauliflowerlike mass in the bottom with the supernatant broth clear but smooth strains may grow more diffusely In glucose agar shake cultures incubated aerobically for 4 to 6 days the typical picture is that of whitish spherical or mulberry shaped colonies of varying size up to 3 mm in diameter growing only in the depths of the agar often with a dense zone of colonies 0 5 to 2 cm below the free surface Recovery of an occasional *A israelii* colony from materials subjected to direct contamination from a mucous membrane (e g in expectorated sputum) has no diagnostic significance

A israelii is sensitive to several antibiotics in vitro (see McVay and Sprunt 1953) Penicillin tetracyclines chloramphenicol and isoniazid have all been reported as effective with occasional failures High dosage for sustained periods is usually used accompanied by surgical measures where possible (See also Peabody and Seabury 1960)

BACTEROIDES AND ANAEROBIC STREPTOCOCCI—MIXED OR SYNERGISTIC ANAEROBIC INFECTIVE DISEASES

A diverse group of diseases of animals and man has been associated for many years with bacteroides or anaerobic streptococci separately or together or mixed with other bacterial species Bacteroides were described in calf diphtheria in 1884 by Loeffler and were found in and cultivated from necrotic disease of other animals in the years immediately following (see Lahelle 1947 Rosebury 1962) Bacteroides were recovered from fetid and gangrenous suppurations in man by Veillon and Zuber in 1897 Veillon also isolated anaerobic streptococci from the lesions of Ludwig s angina perinephric abscess and bartholinitis in 1893 Schottmuller first cultivated anaerobic streptococci from the blood in 1910 These species perhaps occasionally alone more often in bacterial associations are capable of inducing significant disease However the pathogenesis is virtually unknown The clinical processes concerned may be classified in three groups In the first anaerobic or micro aerophilic streptococci occur with other indigenous bacteria in a spreading gangrene of the skin or in wound infections In the second either anaerobic streptococci or bacteroides or both recovered in blood cultures with or without additional species are found in diseases beginning especially in the pharyngeal intestinal or genital mucous membranes and progressing to a septicemia or pyemia often associated with thrombophlebitis In the third group in which indigenous spirochetes are prominently associated with bacteroides anaerobic streptococci and a profusion of other indigenous species—the so called fusospirochetal diseases—the lesions are usually inflammatory or destructive processes of the mucous membranes themselves or of directly adjacent tissues The last group is considered separately in the following section the first two are dealt with here as a unit The distinction between the three groups is not sharp It need hardly be emphasized that collectively these diseases occupy a distinctly higher level of complexity than the endogenous infective diseases considered previously and it is not

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extracts of the latter species prepared by grinding with sand digestion with trypsin or extraction with trichloracetic acid had little or no effect

In vitro antibiotic sensitivity tests with these organisms have been reported most recently by Garrod (1955) and by Lodenkamper and Stienen (1955) The former found *B nigrescens B funduliformis* and a fusiform bacillus to be most sensitive to penicillin but *B fragilis* was penicillin resistant All 4 species were also sensitive to oxytetracycline and chloramphenicol more so to the former erythromycin was effective against *B nigrescens* and polymyxin against the fusiform bacillus and *B funduliformis* Streptomycin and bacitracin were generally ineffective The German workers studied both bacteroides and anaerobic cocci separately and as mixtures Best results were obtained with the tetracyclines but resistant strains were found to nearly all antibiotics tested

Such clinical data as are available are generally consistent with these findings Tetracyclines and chloramphenicol have been used effectively penicillin streptomycin and sulfonamides have usually failed See McHenry *et al* (1961) Tynes and Frommeyer (1962)

FUSOSPIROCHETAL DISEASES

The group of diseases in which indigenous spirochetes appear as prominent components of a profuse mixed biota include some of the most widespread ills of mankind The early literature on fusospirochetal disease has been reviewed by Rosebury (1938) The group includes the periodontal diseases which are treated in the next section the complex of ulceromembranous stomatitis or pharyngitis or equivalent disease of the external genitalia and the extreme form of this process noma comparable putrid necrotic diseases of the respiratory and the intestinal tracts a form of tropical ulcer of the skin and similar lesions of other areas Fusospirochetal disease seems always to be superimposed on tissue damage induced by other agencies including scurvy pellagra inanition or other nutritional disturbances agranulocytosis or radiation injury viral infections including measles and primary herpes simplex and probably more commonly complexes or constellations of factors exemplified in the periodontal disease group

Experimental fusospirochetal disease was produced first in rabbits by Veszpremi in 1905 and 1907 and since repeatedly in guinea pigs and other animals by many workers The lesions have been initiated with whole exudates from any of a series of fusospirochetal diseases in man and are regularly transmissible with the resulting animal lesion exudates inoculated by any of several routes Subcutaneous inoculation of guinea pigs the method most commonly used yields either large necrotic abscesses tending to evacuate and heal or a spreading cellulitis which either heals after belated localization or more typically leads to death in 2 to 7 days In the more severe infections the exudate is serofibrinous it is always foul and contains spirochetes in a complex mixture of other bacteria In animals sacrificed while moribund a cellulitis is found dissecting the fascial planes from the point of inoculation the viscera are somewhat congested and the adrenals are enlarged and hemorrhagic but no visceral infection is found In such animals as in comparable disease in man spirochetes have been found in vital tissue while other bacteria are limited sometimes sharply to the distal border of the necrotic zone Pathologic changes are those of acute inflammation in fatal infection the cellular response is disorderly or at times apparently entirely lacking The species in one guinea pig passage exudate (Rosebury *et al* 1950) in which the LD_{50} was estimated as 8×10^9 organisms were identified as spirochetes of the *T dentium* variety *Fusobacterium fusiforme* and atypical fusiform bacilli both *Vibrio sputorum* and *Spirillum sputigenum* unidentified bacteroides *Str mitis* and incompletely characterized anaerobic streptococci and a miscellany of gram positive bacilli all except *Str mitis* appeared to be strict anaerobes From another passage exudate Macdonald *et al* (1954) isolated a similar range of species but only atypical fusiform organisms were present as was an unidentified motile gram negative anaerobic bacillus and *B nigrescens S sputigenum* was not found A later study by these workers (Macdonald *et al* 1956) indicates that one of the cultures was an aerobic diphtheroid

surprising that the role of indigenous micro organisms in these processes is frequently dis regarded or overlooked The record suggests that these organisms have been found among hospital cases whenever appropriate means have been employed to detect them (See Tynes and Utz 1960 McHenry *et al*, 1961 Johnson 1962, Tynes and Frommeyer 1963 and Murphy *et al* 1963)

The role of anaerobic streptococci and bacteroides in endogenous infective diseases is made clear most convincingly by studies of their experimental pathogenicity and is supported by the results of antibiotic therapy in man The following discussion is limited to these two topics For reviews of other aspects of this subject see Lahelle (1947) Carter *et al* (1953) and Alston (1955)

In pure culture anaerobic streptococci have been found to be nonpathogenic for animals almost uniformly As for bacteroides, on the other hand early workers had little difficulty demonstrating pathogenicity especially with strains isolated from animal disease, but since about 1935 such tests have nearly always resulted negatively Dack (1940) suggested that animals used by the earlier workers may have been fed deficient diets The more significant findings relating to pathogenicity are to be found in studies with mixtures of pure cultures which have yielded positive results consistently when the mixtures contained organisms identified as or resembling *P evolutus* *P putridus* (or incompletely identified anaerobic cocci) *B funduliformis* *B fragilis* or *B nigrescens* Meleney (1931), Henthorne and McDonald (1936) and Steinhorn (1945) have reported that mixtures of anaerobic streptococci with staphylococci or unnamed second species inoculated subcutaneously produced ulcerative, necrotic or gangrenous lesions, often very destructive and sometimes fatal in guinea pigs, mice or other animals, the individual cultures inoculated alone showed little or no pathogenicity Altemeier (1942) produced severe cellulitis with abscess formation ulceration or gangrene in guinea pigs with mixtures containing 4 to 6 pure cultures isolated from peritonitis in man The pure cultures by themselves were again noninfective for the most part Five of the 6 mixtures included either an anaerobic streptococcus or a bacteroides culture, the other species studied included aerobic cocci coliforms *C hofmannii* and *Ps aeruginosa* Ryff and Lee (1946) found that a strain of *B funduliformis* isolated from necrotic stomatitis in a calf produced lesions independently when inoculated in 0 5 ml amounts into the labial skin of rabbits When 0 1 ml of this culture in itself innocuous was mixed with an equal volume of one of a long series of pure cultures of other species most of which were also nonpathogenic independently necrotic or suppurative lesions resulted in the majority of trials Among the ancillary non pathogens were indigenous aerobic streptococci, micrococci or corynebacteria *B subtilis* and *L bifidus* In additional experiments by these workers more severe lesions resulted in rabbits fed deficient diets and then inoculated similarly with the bacteroides cultures mixed with an aerobic streptococcus or *B subtilis* The most extensive studies of this kind were reported by Hite *et al* in 1949 These workers used a group of cultures isolated from the female genital tract under either normal or pathologic conditions including *B funduliformis* *B nigrescens* and other bacteroides, a strain listed as *Bact fusiformis* but not otherwise identified a group of anaerobic streptococci and other cocci not clearly characterized and the aerobes *Str liquefaciens* *Str mitis* and *Staph albus* Subcutaneous inoculation of mice with single pure cultures of the anaerobes again yielded minimal lesions or none the aerobes alone were nonpathogenic In pairs however, comprising either two anaerobes or anaerobe plus aerobe inoculation of half volumes of each yielded lesions with many of the mixtures described as necrotizing often suppurative sometimes extending to the peritoneum and the viscera and in some instances leading to death of the animal The most significantly pathogenic mixtures contained anaerobic cocci with either *Str liquefaciens* or the fusiform bacillus bacteroides with any of the aerobes or, curiously *B funduliformis* with either *B nigrescens* or the fusiform bacillus It is of interest that mixtures of bacteroides with the anaerobic cocci were generally ineffectual Synergistic infection was also observed when *Str liquefaciens* was mixed with heat killed *B funduliformis* but mixtures of the same streptococcus with

cal massive overgrowth of the fusospirochetal biota is present in all three varieties and is indistinguishable among them The inflammatory and destructive symptoms of periodontal disease are referable to fusospirochetal infection which in turn may be traced to tissue damage associated with gingival accumulations of food desquamated epithelium and salivary calculus the first two serving as pabulum for the biota the last presumably as an additional source of irritation and tissue deformation These antecedents of infection may in turn be attributed to a wide range of subtle local or general disturbances in the structural and functional relationships of the dental periodontal apparatus

The pattern of ideal relationships entails a complex equilibrium changing with time between functional stress upon the teeth occlusal tooth wear and continued eruption of the teeth and apposition of cementum probably accompanied by a rootward shift of the terminal epithelium of the gingiva Functional stress transmitted through the teeth maintains the integrity of the periodontal ligament and the alveolar bone and both structures shift and change with age to compensate for cusp loss and interproximal attrition Additional compensatory changes with age involve these tissues the dental arches as units and the temporomandibular joints Disturbances in this process which alter the ideal relationships of the attached epithelium the gingival margin and the underlying bony support impair the hygienic mechanism whereby ideally functional movements prevent gingival accumulations of gross bacterial pabulum such accumulations and the consequent infection aggravate the cycle of changes

The circumstances that induce periodontal disharmony are themselves varied and complex They may include any agency that leads to impaired cell metabolism of the tissues concerned nutritional or hormonal upsets intoxications or exogenous infections abetted by tooth loss or damage resulting from dental caries or by faulty dental restorations and doubtless also by the character of ingested food as it promotes or fails to promote healthy function and retention of food residues in addition to its nutritional quality Emotional disturbances interrelated with metabolic upsets and with salivary function stimulated by gross fusospirochetal infection clenching and grinding of the teeth neglect of nutrition and toothbrushing must also be included among the forces leading to periodontal disease It is probable that the customary diet of the present day promotes a marginal gingivitis if it is inadequately compensated for by toothbrushing this form of the disease usually responds quickly to simple hygienic treatment If treatment is deferred the cycle of changes proceeds to suppurative periodontal breakdown which can usually be arrested by similar treatment of the more deeply involved tissues Under more acute stress such as fatigue intoxication and deficiency especially of B group vitamins the ulcerative form of gingivitis may appear Outbreaks of this condition have occurred under group conditions especially in wartime It is also likely that a metabolic disturbance such as a transient deficiency of ascorbic acid may initiate a cycle of changes in the periodontal tissues that eventuate in breakdown many years later Ascorbic acid deficiency and consequent failure of collagen formation lead to a condition in guinea pigs that resembles acute periodontal breakdown Such metabolic disturbances are particularly implicated in a form of periodontal pocket formation that is not accompanied by gingivitis

Periodontal disease has been observed or produced experimentally in several animal species (see Gupta and Shaw 1956 Baer and White 1960 Macdonald *et al* 1960) but findings thus far have shed little light on the problem in man Alveolar bone loss one of the features of chronic periodontal disease in man has been found in germ free mice by Baer and Newton (1959) Treatment of periodontal disease varies with the clinical entity Chemotherapy is indicated only to relieve acute symptoms e g in ulcerative gingivitis recurrence is likely unless other measures are employed Such measures emphasize the removal of gingival and subgingival microbial pabulum and tartar and where necessary the correction of faulty dental restorations and of defects of occlusion and alignment of the teeth Local treatment of this kind supplemented by toothbrushing is frequently sufficient in itself to arrest the dis

Socransky *et al* (1963) and Gibbons *et al* (1963) have reported quantitative studies of the gingival microbiota in which the micro organisms were similar in health and in peri odontal disease except for a significant in crease in spirochetes in the latter there was more than a 10 fold increase of the total flora in disease

Attempts to reproduce transmissible fuso spirochetal infections with recombined mix tures of pure cultures and thus to define the essential component species have yielded equivocal results up to the present The earlier studies initiated by Kritchewski and Seguin in 1920 have been reviewed by Rose bury *et al* (1950) D T Smith in 1932 and Proske and Sayers in 1934 reported that a mixture of pure cultures of 4 anaerobes —*T microdentium* a fusiform bacillus a vibrio and a streptococcus—was the only effective combination among many tested Rosebury *et al* (1950) using the cultures previously noted and others were unable to produce typical or transmissible fusospiro chetal infection with similar or other com binations However typical infection could be induced by inoculation of cultures grown from whole exudate as a mixture through 10 successive culture passages without loss of infectivity hence the infective mixture is cultivable in vitro as such An estimate of the dilution of the original inoculum entailed by this procedure was in line with other studies which suggested that a virus is not a com ponent of the infective complex Successful recombination was accomplished by Mac donald *et al* (1954) who produced typical transmissible fusospirochetal infection with material taken from the hub of wheel plates' on which 16 cultures isolated from guinea pig exudate had been streaked radially to a common center spirochetes from a cul ture having been added at the hub In 3 of 9 trials in which the spirochetes and all or all but one of the spoke cultures grew under these conditions an emulsion of the mixture reproduced the typical disease In a subse quent study Macdonald *et al* (1956) using the wheel plate method recombined the same 16 cultures in various groups and concluded that the minimum group capable of eliciting typical transmissible infection consisted of 2 strains of *Bacteroides* (including *B nigrescens*), an unidentified motile gram negative anaerobic bacillus and an aerobic diphther oid The diphtheroid was thought to serve the nonspecific function of supporting growth of *B nigrescens* None of the species con sidered essential by D T Smith and Proske and Sayers was present in the inoculum nor did any appear in the exudates from which the 4 species employed were reisolated The positive aspects of these findings are in line with those noted previously in experimental studies of bacteroides but their negative as pects as Macdonald and his co workers themselves suggested and as Berger (1957) has pointed out cannot be considered as demonstrating that neither spirochetes nor typical fusiform bacilli participate in fuso spirochetal infections

The probability that bacteroides and fuso spirochetal diseases although pathologically similar may be etiologically distinct is sup- ported by findings with penicillin therapy It has been noted that *B fragilis* is resistant to penicillin in vitro and that although other bacteroides are penicillin sensitive clinical trials with this antibiotic have yielded poor results The value of penicillin in controlling the infective phase of fusospirochetal disease in man on the other hand was established early and has included a wide range of fuso spirochetal diseases among them the highly destructive and fatal forms in agranulocytosis (Robertson 1949) and noma (Jelliffe 1953)

PERIODONTAL DISEASE

The following brief treatment of periodon tal disease is included here for its intrinsic interest and as an example of the presumed interplay of host factors in an endogenous infective disease The three principal varie ties of periodontal disease comprise a sub acute or chronic inflammation of the gingival margin (marginal gingivitis) an acute ulcer ative variety (Vincent s gingivitis) and perio- dontal breakdown (pyorrhea) the most chronic form characterized by progressive development of pockets opening at the inner gingival margin Chronic periodontal disease occurs nearly universally in the 4th or the 5th decade of life and is mainly re sponsible for tooth loss among adults A typi

cal massive overgrowth of the fusospirochetal biota is present in all three varieties and is indistinguishable among them The inflammatory and destructive symptoms of periodontal disease are referable to fusospirochetal infection which in turn may be traced to tissue damage associated with gingival accumulations of food desquamated epithelium and salivary calculus the first two serving as pabulum for the biota the last presumably as an additional source of irritation and tissue deformation These antecedents of infection may in turn be attributed to a wide range of subtle local or general disturbances in the structural and functional relationships of the dental periodontal apparatus

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ease Surgical procedures for elimination of pockets and diseased gingival tissue are widely and successfully used For additional details and references see Rosebury 1952 *1955 Glickman 1956 1963*

DENTAL CARIES

Dental caries one of the most prevalent of all diseases is a distinctive process of disintegration of the hard dental tissues proceeding centripetally from the exposed surface Its unique characteristics are determined in part by the tissues in which it appears which are either entirely noncellular (enamel) or contain only the processes of pulpal cells (dentin) and hence do not react to injury as do cellular tissues Most carious lesions begin at one of three sites in the pits and fissures of the functional surfaces at approximating areas of contact and on surfaces of enamel or exposed cementum or dentin near the gingival margin Lesions in the first two areas are most common in the period immediately following tooth eruption, i e, in childhood and adolescence There is nearly universal agreement on the main lines of pathogenesis stemming principally from the studies of W D Miller in 1890 Research during the last decade has generally supported this approach while elucidating many details (e g biochemical and histochemical studies Mandel, 1955 Opdyke 1962 Zipkin and Gold 1963 microradiography Bergman and Engfeldt 1954 Guzman *et al* 1957 electron microscopy Scott and Albright 1954 Helmcke 1959 and in vitro experimental caries Forziati *et al* 1956 Rowles *et al* 1963)

According to this view caries is initiated by acid products of bacterial fermentation of carbohydrates formed locally at sites of food accumulation or impaction Demineralization of enamel (or of dentin or cementum if the lesion begins in these tissues) appears to be the first step in the process subsequent steps may vary in seqeunce depending in part on the site and on the rate of progress of the lesion They include bacterial invasion proteolysis of enamel matrix, decalcification followed by digestion of dentin or cementum and loss of tissue by minute or gross fracture consequent on both function and forces associated with demineralization such as shrinkage and gas formation The bacterial species involved are almost certainly mixed in all instances but lactobacilli probably play a distinctive role in the mixture they alone among bacterial species recovered from caries have been found to be significantly increased in relation to clinical caries activity and their ability both to produce and to withstand low pH levels is unlikely to be coincidental The failure of germ free rats given sterilized caries producing diets to develop lesions (Orland 1954) confirmed the indispensability of microorganisms in the process Keyes (1960) has reported that experimental caries in hamsters and rats is infectious and transmissible under laboratory conditions but applicability of these findings to man is doubtful Whether the finding that rodent caries can be initiated specifically by certain streptococci (Fitzgerald and Keyes 1960 Keyes and Fitzgerald 1962) can be applied to man is also conjectural (Fitzgerald 1963)

For the most part our knowledge of the pathogenesis of caries is consistent with the fact that the disease is nearly universal in modern man Its apparently essential ingredients—retentive sites on the teeth the participating microorganisms and the required pabulum as well as the susceptibility of the teeth to disintegration all appear to be nearly ubiquitous Yet the occasional complete absence of caries in an adult may be attributable in part to the first factor, tooth form and dental architecture presumably implying a genetic component and the most probable mechanism for the protective effect of fluoride ion seems to be related to the last item the acid solubility of the dental tissues Nevertheless wide individual variation both in prevalence and in rate of progress of caries examined in detailed context points to the operation of additional factors and provides at least potentially means toward control

Experimental caries in hamsters and certain experimental lesions in rats seem to be most comparable with dental caries in man From the studies of Rosebury *et al* (see Rosebury 1938b) Sognnaes (1948) and Gustafson *et al* (1953 1955) emerge a group of interdependent determinants of caries susceptibility that seem to be consistent

with the human disease These include (1) the retentiveness of carbohydrate foods as determined by their physical state (2) the metabolic availability of the carbohydrate as substrate for fermentation (3) dental function or lack of function as it promotes or prevents food retention (4) a protective role of dietary fat perhaps acting by retarding carbohydrate solubility and (5) many other conditions less clearly defined including nutritional endocrine and other factors affecting the structure of the dental tissues salivary flow and composition and the pabulum retaining propensities of periodontal (interproximal and gingival) areas There is cogent evidence that starchy foods in dry compact form (e g raw rice or corn in animals hard fat free biscuit in both man and rats) serve as the initiating substrate for caries by forcible impaction into retentive sites On the other hand certain sugars especially sucrose and fructose but not starch or dextrin incorporated in the diet in dry finely divided form promote caries particularly in hamsters in proportion to their concentration In this instance it appears that impaired function evidenced after extraction of opposing teeth is necessary to permit accumulation or prevent removal of the pabulum When to such sugar diets enough distilled water was added to dissolve the carbohydrate caries in hamsters was markedly reduced suggesting that the major cariogenic effect of the diet is exerted in the mouth and not via nutritional channels (Gustafson *et al* 1955) Retardation of caries by inclusion of fat in the diet first noted in studies with children by Mellanby *et al* (see Committee 1936) has been observed in rats by Rosebury and Karshan (1935 1939) and Granados *et al* (1948) and in hamsters by Gustafson *et al* (1955) These findings suggest the possibility of preparing noncariogenic carbohydrate foods perhaps even confections by appropriate admixture with fats Among other influences on caries it may be noted that abundant evidence from studies both in man and in experimental animals indicates that if basic cariogenic conditions are present nutritional adequacy e g of vitamin D decreases or retards the progress of the lesions

Diagnosis of dental caries is made clinically with the aid of roentgenograms Counts of lactobacilli in saliva and other caries activity tests are useful principally in confirming a clinical diagnosis of rampant caries and as a sensitive index in the individual patient of the progress of attempts at dietary control Control of unusually high caries activity in children has been effective by drastic limitation of carbohydrate intake The use of a variety of dentifrices containing alleged anticariogenic agents is of doubtful utility but carefully supervised toothbrushing with any dentifrice or none may have some value Caries can be arrested by the traditional dental practice of removing involved tissue and replacing it with inert filling materials The fluoridation of public water supplies to the average level of 1 part per million has been fully established as nontoxic and effective in reducing the community prevalence of caries by 60 per cent or more most actively in children born and reared on fluoridated water but with distinct value when started at any age (see Arnold 1957 Hayes Littleton and White 1957) This now widespread practice is probably the most thoroughly studied of all public health procedures its effectiveness is beyond serious question but it is capable of solving only approximately half the problem of dental caries The effectiveness of topical application of 2 per cent NaF to the teeth of children has also been clearly demonstrated and is applicable where community fluoridation is not feasible The mechanism of inhibition of caries by fluoride seems to involve its incorporation in enamel as fluorapatite

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15

The Hemolytic Streptococci

The streptococci comprise a large and biologically diverse group of microorganisms. The principal criteria for classifying them are morphologic. In general, streptococci can be defined as gram-positive organisms, spherical or oval in shape, that tend to grow in chains. With the exception of the pneumococci, which are usually considered separately by medical bacteriologists because of their unique biologic characteristics, those streptococci which possess primary pathogenicity for man and animals belong to the subdivision of hemolytic streptococci. The nonhemolytic streptococci are saprophytic, and many species can be found among the flora of the upper respiratory and the intestinal tracts of normal individuals. They are sometimes found in association with infectious processes, most commonly when some predisposing structural abnormality is present, as in bacterial endocarditis, urinary tract infections, or secondary wound infections.

Hemolytic streptococci cause the lysis of mammalian red blood cells, a property which is useful in the initial isolation and identification of these organisms in culture material. As a result of extensive serologic studies they have been divided into a number of distinct serologic groups which are related in some degree to the natural habitat and the pathogenicity of the organism. Accordingly, serologic group has assumed primary importance in the identification of pathogenic streptococci. It has become apparent that hemolytic streptococci of a single group, group A, are not only responsible for most of the acute streptococcal disease of man but also are involved in the initiation of late sequelae such as rheumatic fever and glomerulonephritis.

HISTORY

Streptococci were first seen in materials obtained from certain human infections. Thus Billroth (1874) described globular chains of microorganisms in erysipelas and wound infections, and Pasteur (1879) demonstrated similar organisms in the blood of a patient with puerperal sepsis. Ogston (1881) isolated cocci growing in chains from acute abscesses and used animal experiments to demonstrate the pathogenicity of the organisms after they had been grown on artificial media. Following the demonstration by Koch (1881) that streptococci were present with great regularity in excised erysipelas lesions, Fehleisen (1882, 1883) grew the organism in pure culture from such areas and induced typical erysipelas in human beings with subcultures.

Organisms with similar characteristics were subsequently obtained from a wide variety of materials, and considerable difficulty arose concerning their identification and classification. Inevitably, many streptococci were considered to be specific for the disease entity in which they appeared, and as a result such names as *Streptococcus erysipelatis* and *Streptococcus scarlatinae* came into use. Attempts to arrive at a system of classification

by studying biochemical and physiologic properties such as the pattern of fermentation by various carbohydrates did not prove to be of great value since no clear relationship between these findings and disease producing capacity could be established The differentiation of streptococci into hemolytic green and indifferent strains based on their action on red blood cells in vitro was first proposed by Schottmuller (1903) In his elaboration of this technic Brown (1919) introduced the terms alpha beta and gamma to describe slight hemolysis with greening frank hemolysis or no effect on erythrocytes respectively It was recognized early that pathogenic streptococci for the most part fell in the truly hemolytic (beta) category

A useful system for the classification of hemolytic streptococci was gradually evolved by the application of serologic technics Although not historically the first step a development of primary importance in bringing order into the study of streptococci came with the separation of these organisms into well defined serologic groups by Lancefield (1933) It was clear that most strains associated with human infections belonged to a single serologic group designated group A The occurrence of serologically differentiable types among human strains had been demonstrated earlier by agglutination and mouse protection tests (Dochez Avery and Lancefield 1919) The further development of type differentiation of human strains was pursued by different approaches in 2 laboratories Lancefield (1928a) devised a precipitin technic for typing based on the use of extracts containing type specific protein antigens and Griffith (1927 1934) classified streptococcal strains by applying a slide agglutination technic Information obtained from the grouping and the typing of hemolytic streptococci established the fact that the use of disease specific names is not justified since group A strains of a single type can be recovered from a variety of disease entities More important the serologic classification of streptococci supplied the tools required for a detailed study of the bacteriology the immunology and the epidemiology of streptococcal disease

MORPHOLOGY

The outstanding morphologic characteristic of streptococci is their tendency to grow in chains At certain stages of the growth cycle the individual cocci are spheroid However during growth of the organisms prior to division they become elongated on an axis parallel with that of the chain and assume an ovoid appearance The average diameter of the cocci is approximately 1 micron although there is considerable variation in size and certain minute streptococci may be one fourth to one half this size When growing under unfavorable conditions bizarre forms frequently appear with irregularities in the size the shape and the gram positivity of cocci even within a single chain In young actively growing cultures in favorable media such as serum or blood broth the cocci are uniform and the chains appear in their most characteristic form

After elongation during growth the cocci divide in a plane perpendicular to the long axis of the chain Because the individual cocci divide into pairs a diplococcoid appearance of the members of a chain is often quite striking Chaining results from the fact that a connecting link between the cocci probably composed of material like that forming the cell wall is retained following division These intercellular bridges are not easily broken and withstand such procedures as shaking however the chains can be disrupted without extensive destruction of cocci by subjecting them to brief periods of sonic oscillation (Slade and Slamp 1956) The length of the chains varies within wide limits and is influenced to some degree by the nature of the culture medium Many hemolytic streptococci grow in relatively short chains of 8 to 10 members others much longer Occasionally one will find extremely long chains suggesting that each individual chain of the inoculum has continued to increase in length without disrupting during growth This type of chain formation is more common among certain of the nonhemolytic streptococci

The chain forming property has an important bearing on the interpretation of quantitative studies on the growth of streptococci The usual technic for determining the number of viable bacterial cells depends on count

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composition are formed by certain members of group C These capsules are composed largely of hyaluronic acid which is either identical with or very closely related to the hyaluronic acid found in mammalian tissues Capsules are most readily demonstrated microscopically in very young actively growing cultures in enriched media With continued growth the hyaluronic acid is released into solution in the medium and capsules are no longer demonstrable The capsules are seen most easily in wet preparations prepared by the India ink technic and appear as a large clear zone surrounding the chains frequently several times the diameter of the cocci

Capsulation in group B streptococci is quite different in nature and depends on the occurrence of type specific polysaccharides comparable with those elaborated by pneumococci

COLONY FORMS

The colonial characteristics of hemolytic streptococci are usually studied on the surface of blood agar plates so that the pattern of hemolysis and colony morphology can be observed simultaneously The size the distinctness and the rate of development of the zone of hemolysis surrounding surface colonies vary considerably Some strains notably certain members of groups C and D produce unusually large and brilliant zones while others may show no more than a narrow ring of hemolysis surrounding the colony The species of blood used in the agar medium influences the nature of the hemolysis and streptococci occur which give clear hemolysis with one mammalian blood but cause no hemolysis or only greening with others

Not all streptococci which fall into the recognized serologic groups produce hemolysis on the surface of blood agar plates Thus certain members of groups B C D H K and O and all members of group N are essentially nonhemolytic Even among group A strains variants occur which lack the ability to produce or release streptolysin S and thus are nonhemolytic under the aerobic conditions of surface growth Variants of this type are being encountered with increasing frequency on routine throat cultures leading to some difficulty in the initial recognition of pathogenic strains Such strains possess the colonial morphology of typical group A strains and produce the oxygen labile streptolysin O so that they become hemolytic when grown anaerobically or as deep colonies in agar pour plates

Isolated colonies of streptococci on blood agar average 1 to 2 mm in diameter The so called minute streptococci including members of groups F and G form much smaller colonies The topography of the colonies does not follow a single well-defined pattern they are usually circular in outline with smooth or slightly serrated edges and a rounded surface

Among group A streptococci several different colony forms are recognized and the descriptive terms most commonly employed are mucoid matt and glossy The mucoid character is associated with the production of hyaluronic acid capsules and the most strikingly mucoid strains are those that form abundant amounts of polysaccharide with large well-developed capsules Mucoid colonies are observed most satisfactorily on fresh blood agar plates or plates that have been sealed to prevent loss of moisture They tend to be large and glistening and may have the appearance of droplets of water (Fig 1 A) although when touched with a loop the viscous character of the colony is readily apparent The suggestion of fluidity is increased by the fact that the colonies frequently do not retain a circular shape The hyaluronate gel often collapses as a result of drying enzymatic action or chemical effect leaving a flattened colony with an irregular surface (Figs 1 B and C) It is these colonies that are currently referred to by the term matt although they might more appropriately be designated as "postmucoid

Glossy colonies are formed by strains that do not produce capsules during growth on agar These nonmucoid colonies are smaller and usually more rounded than the mucoid forms and they tend to have a shiny reflecting surface (Fig 1 D) The importance of the capsule in colony form is illustrated by the fact that strains such as those shown in Figures 1 A to C produce typical glossy colonies when grown in the presence of hyaluronidase

There is a limited relationship between colony form and production of antigenic constituents responsible for type specificity and

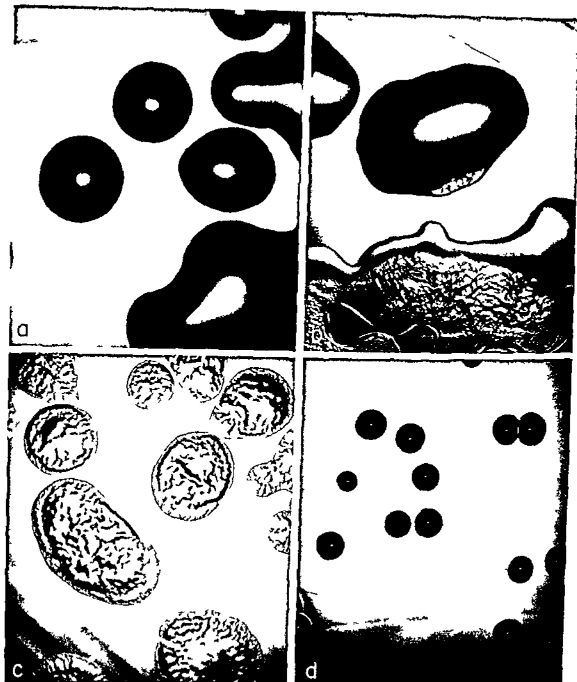


FIG 1 Colony forms of group A streptococci Magnification 5 5 × a Mucoid colonies b Mucoid colonies with beginning conversion to post mucoid state c Postmucoid colonies d Nonmucoid (glossy) colonies (Photographs from Dr A T Wilson Alfred I du Pont Institute of the Nemours Foundation Wilmington Delaware)

ing the colonies formed when dilutions of the culture are incorporated in an appropriate agar medium In the case of streptococci this procedure provides an estimate not of the number of individual viable cells but of the number of intact chains

Capsule formation is characteristic of many group A streptococci and capsules of similar

the amount of growth significantly The reducing conditions necessary for the initiation of growth in these media are usually obtained by the addition of a sulfhydryl compound such as cysteine or thioglycollate

There is a certain amount of evidence to suggest that the missing substances which account for the inadequacy of defined media are peptide in nature The substance designated streptogenin (Woolley 1941) which was found to be necessary for the growth of certain group A streptococcal strains is apparently a family of peptides rather than a single substance Others have found that peptide preparations are stimulatory to the growth of group A streptococci in defined media (Slade 1954)

Theoretically investigation of the extracellular enzymes and toxins of group A streptococci would be carried out most satisfactorily with defined media because of the absence of macromolecular constituents not arising from the organisms However these media have proved to be generally unsatisfactory for this purpose and the alternative approach of employing only the dialyzable components of complex meat infusion peptone media has been adopted (Stock 1939 Dole 1946) Most strains grow well and produce the expected complement of extracellular substances in appropriate dialysate media

The hemolytic streptococci are facultative anaerobes and metabolize glucose with the formation of lactic acid The accumulation of lactic acid with the attendant fall in pH is a limiting factor in the growth of the organisms in an otherwise adequate medium as indicated by the fact that massive growth can be obtained in the presence of excess glucose if the lactic acid formed is continuously neutralized Growth is optimal at 37 C and is markedly inhibited at temperatures above 40 C

GROUP DIFFERENTIATION

The division of hemolytic streptococci into serologic groups depends on the occurrence of group specific cellular antigens which are carbohydrate in nature and are designated as C carbohydrates (Lancefield 1928b 1933) The presence of these antigens is demonstrated by means of precipitin reactions between solutions of the carbohydrate extracted from streptococcal cells and antisera prepared by immunizing rabbits with heat killed suspensions of streptococci Lancefield showed that separating hemolytic streptococci into distinct categories by this technic resulted in a classification related to the most characteristic source of the organisms Thus group A is composed of strains usually pathogenic for man group B of strains from bovine mastitis group C is commonly found in streptococcal diseases of lower animals etc The source and the characteristics of the common serologic groups—including those that have been added since the initial studies of Lancefield—are summarized in Table 1 The relationship between group specificity and habitat is not a rigid one Thus although group A strains are responsible for most human streptococcal infections occasionally representatives of all other groups may be isolated from human sources

The serologic groups listed in Table 1 embrace the great majority of strains of hemolytic streptococci However additional strains are encountered which possess the general properties of hemolytic streptococci but do not fall into any of the defined groups

Information concerning the cellular localization and the chemical composition of the group specific carbohydrates has been obtained in recent years In the case of group A streptococci the carbohydrate is an integral component of the bacterial cell wall and may account for as much as 10 per cent of the dry weight of the organism (McCarty 1952 Salton 1953) The same localization has been shown to hold for several other groups (B C G and E) and the presence of rhamnose as a major monosaccharide in the cell wall of all major groups of streptococci indicates a uniform pattern which supports the concept of a basic interrelationship between the hemolytic streptococci (Cummins and Harris 1956 Slade and Slamp 1962) Variations in the content of hexosamines and other monosaccharides in the cell wall of different groups provide an adequate chemical basis for serologic differences

The group D streptococci stand as an important exception since the group specific antigen in these organisms has been identified as a member of the family of glycerol

TABLE 1 SUMMARY OF RECOGNIZED SEROLOGIC GROUPS OF STREPTOCOCCI

GROUP	USUAL HABITAT	USUAL PATHOGENICITY
A	Man	Many human diseases
B	Cattle	Mastitis
C	Many animals	Many animal diseases
	Man (human strains)	Mild respiratory infections
D	Dairy products	Genitourinary tract infections endocarditis wound infections
	Intestinal contents of man and animals (enterococci)	
E	Normal milk	None known
	Swine	Pharyngeal abscesses of swine
F	Man	Questionable found in respiratory tract
G	Man	Mild respiratory infections rare
	Dogs	Genital tract infections in dogs
H	Man	Questionable found in respiratory tract
K	Man	Questionable found in respiratory tract
L	Dogs	Genital tract infections
M	Dogs	Genital tract infections
N	Dairy products	None
O	Man	Occur in upper respiratory tract but not associated with disease Endocarditis

Groups A to E were described by Lancefield (1933) groups F and G by Lancefield and Hare (1935) groups H and K by Hare (1935) and groups L and M by Fry (unpublished) Group N was identified independently by several groups and the letter N was assigned by Shattock and Mattick (1943) Group O was described by Boissard and Wormald (1950)

virulence Strains that produce mucoid colonies usually elaborate M protein and are potentially virulent while those that produce glossy colonies often contain little or no M protein and are of low virulence However there are many exceptions to this relationship and colony form cannot be used as a reliable indication of either M protein production or potential virulence Thus nonvirulent strains can produce hyaluronic acid and give mucoid colonies and M protein may be produced by organisms which form typically glossy colonies These relationships have been discussed in detail by Wilson (1959)

Other more subtle differences in colony topography between strains of group A streptococci can be demonstrated when the organisms are grown on special media and examined at higher magnification but the basis for these differences is not yet established

GROWTH REQUIREMENTS

The pathogenic hemolytic streptococci are among the most fastidious of microorganisms in growth requirements As a routine procedure they are usually cultivated in complex media composed of peptone meat infusion salts and glucose Heating may reduce the ability of a medium to support growth and the best results are obtained when the broth is sterilized by filtration With even the most satisfactory media of this type further enhancement of growth usually can be achieved by the addition of blood or serum The effect of blood or serum is especially striking in the case of solid media since the growth of streptococci on the surface of agar plates tends to be quite poor in the absence of these additives

Some information concerning the minimal requirements for the growth of group A streptococci has been obtained by the use of chemically defined media (Bernheimer *et al* 1942 Slade *et al* 1951) although very few strains will grow vigorously and reproducibly under these conditions The findings indicate that up to 15 amino acids and almost all of the known members of the vitamin B complex are needed In addition purines and pyrimidines are required for certain strains and asparagine and glutamine may increase

different methods have been employed for this purpose (1) Extraction of cells at pH 2 and 100 C (Lancefield 1928a b) This method has the distinct advantage of simul taneously providing M protein containing ex tracts suitable for typing of group A strains (2) Extractions of cells with formamide at 160 to 180 C (Fuller 1938) (3) Lysis of streptococci with enzymes derived from *Streptomyces albus* (Maxted 1948) The cell wall is actually dissolved by this proce dure consequently the highest yields of car bohydrate are obtained (4) Autoclaving of suspensions of cells at 15 pounds pressure for 15 minutes (Rantz and Randall 1955) Characteristically solutions of the carbohy drate react rapidly with specific antisera to give floccules of antigen antibody precipitate and show little or no cross reaction with heterologous group antisera Thus with an appropriate supply of antisera the serologic group of unknown strains can be quickly determined The glycerol teichoic acid anti gens are also released in serologically active form by these several methods

Serologic grouping can also be achieved without extraction of the antigen by appli cation of the fluorescent antibody technic (Moody *et al* 1958) When carried out with reliable reagents and adequate controls on streptococci isolated from throat cultures this procedure gives results comparable with those obtained by the precipitin test

A nonserologic method of differentiating group A from the other groups of strepto cocci has been suggested by Maxted (1953) Group A strains were shown to be signifi cantly more sensitive to bacitracin than strains of all other groups and by choosing the appropriate concentration of the anti biotic Maxted devised a simple plate method for tentative identification of group A strains In a survey of a large series of strains the accuracy of this technic proved to be very high although rare exceptions were encoun tered

Streptococcal bacteriophages have been known for many years but the earlier studies were carried out for the most part without reference to serologic grouping Recent studies have shown that several group A phages are related serologically to one an other but distinct from group C phage fur thermore that susceptibility of streptococci of these two groups is primarily a group specific phenomenon (Krause 1957) al though other factors influence the suscepti bility of group A strains (Maxted 1955) The cell wall carbohydrate of group C strains inactivates group C phage suggesting that the group specific carbohydrate may serve as a phage receptor site in this case but no similar effect can be demonstrated with group A carbohydrate Also of interest with respect to the cell wall structure of streptococci is the fact that phage lysates of group C strep tococci contain a lytic factor presumably an enzyme which is highly active on group A and group E strains as well as on group C (Maxted 1957 Krause 1957) This en zyme dissolves the cell wall with release of the group specific carbohydrate in much the same way as the *Streptomyces albus* en zymes

ANTIGENIC AND BIOCHEMICAL COMPOSITION OF GROUP A STREPTOCOCCI

Because of the importance of group A streptococci in human infections the com position of these organisms has been studied in great detail Of particular importance are the several surface antigens and the wide variety of extracellular products which are released into the environment during growth Not all of these antigens are limited in their occurrence to group A streptococci and sub- stances similar to or identical with certain of these components are produced by some members of other groups

Surface Antigens

The properties of the several surface com ponents of streptococci are summarized in Table 2 Most of these are antigenic but the nonantigenic substances in the surface complex are included to provide a complete picture

Group specific Carbohydrate As noted above the group A carbohydrate is the major component of the bacterial cell wall The monosaccharide constituents are rhamnose and N acetyl glucosamine in a ratio of ap proximately 2 1 (Schmidt 1952 McCarty 1952 Salton 1953) When the carbohy

TABLE 2 SURFACE COMPONENTS OF STREPTOCOCCI

	COMPOSITION	DISTRIBUTION	SIGNIFICANCE
Capsule			
Hyaluronic acid	Repeating polymer of N acetyl glucosamine and glucuronic acid	Capsular material in most strains of group A and some of group C	Antiphagocytic effect under certain conditions Human serum contains factor counteracting this effect
Polysaccharide	Complex polysaccharides	Capsular material in group B	Type specific antigens in group B with antiphagocytic effect
Cell Wall			
M proteins	Protein	Type specific antigen on surface of cell wall in group A	Primary factor in virulence of group A streptococci through antiphagocytic effect
T & R proteins	Protein	Also on surface of cell wall of group A strep Analogous proteins probably occur in other groups (e g groups C and G)	No demonstrable role in virulence Participate in agglutination reactions
Carbohydrate	Polysaccharides with rhamnose as prominent component in most groups Hexosamines and often hexoses also present	Integral part of insoluble cell wall probably lying under surface proteins	Group specific antigens of groups A B C E and G (and probably others)
Mucopeptide	Polymer of N acetyl glucosamine and N acetyl muramic acid with peptides attached through carboxyl of muramic acid	Inner layer of cell wall Similar in all groups and in other bacteria	Rigid skeletal framework of cell wall
Glycerol teichoic acids	α Glycerophosphate polymers with additional substituents (sugars D alanine)	Localization unknown Not attached to wall by primary bonds but near surface of cell	Group specific antigens in groups D and N Heterophile antigen present in most groups
Cell membrane	Complex lipoprotein structure	Underlies cell wall	Limiting membrane in L forms and protoplasts Site of synthetic mechanisms for hyaluronic acid (and possibly other surface components)

teichoic acids which are not bound to the cell wall (Elliott 1962 Wicken *et al* 1963) In this instance the rhamnose containing carbohydrates of the cell wall have been designated as type specific antigens so that group D appears to comprise a rather diverse group of organisms all of which produce similar teichoic acids (Elliott 1960) This situation is not confined to a single group and the group-specific antigen of group N streptococci has also been identified as a glycerol teichoic acid

The occurrence of carbohydrate antigens as a part of the essentially insoluble cell wall structure explains the need for relatively drastic procedures in obtaining solutions of the carbohydrates It is necessary to bring about partial disintegration of the cell wall with the release of soluble carbohydrate in a form that retains its serologic reactivity In practice 4

protein is not a highly antigenic substance and prolonged immunization with suitable streptococcal vaccines is often necessary in order to produce adequate amounts of precipitating antibody The antisera obtained must be absorbed with organisms of heterologous types to remove antibodies to common streptococcal antigens which might give cross reactions Because of these considerations typing of streptococci is not widely used as a routine bacteriologic diagnostic procedure However it has proved to be of great importance in special clinical and epidemiologic studies

The detection of antibody to M proteins in human sera poses a special problem The ordinary in vitro tests such as the precipitin reaction are not sufficiently specific for this purpose since M antigen preparations contain other streptococcal substances which give rise to a high incidence of cross reactions As a result the measurement of type specific antibody has depended on complex biologic tests based on the role of the M antigen in virulence and phagocytosis For example it is often possible to demonstrate type specific protection with human sera in mouse infections but this procedure is somewhat insensitive and requires relatively large amounts of M antibody The most widely used procedure has been the so-called bactericidal test which exploits the fact that usually streptococci are rapidly killed following phagocytosis Streptococci containing M protein resist phagocytosis by human leukocytes and grow vigorously in normal blood However in the presence of type-specific antibody phagocytosis is greatly enhanced and virulent streptococci inoculated into blood if not present in excessive numbers can be completely destroyed by leukocytes Quantitative procedures for carrying out phagocytic tests have been devised and have been applied not only to the measurement of M antibody (Kuttner and Lenert, 1944 Rothbard 1945) but also to the antigenic analysis of streptococci (Maxted 1956 Lancefield 1957)

Because of the complexity of the phagocytic test other procedures have been sought for the measurement of type specific antibody Stollerman *et al* (1959) have shown that growth in the presence of M antibody significantly enhances the chain length of selected M-containing strains and have used this phenomenon in the measurement of type specific antibody Efforts have been made to apply such methods as passive hemagglutination in which tanned erythrocytes are treated with M extracts but it has been difficult to achieve specificity because of cross reacting antigens in the extracts which are common to many types of streptococci Some success has been reported recently in the elimination of these cross reactions by inhibition with heterologous extracts (Vosti and Rantz 1964)

Other Surface Protein Antigens In addition to the M protein there are other protein antigens which occur at or near the cell surface and apparently are attached to the cell wall Those which have been studied in detail and are characterized to some degree serologically and chemically have been designated T antigen and R antigen (Lancefield 1940 Lancefield and Perlmann 1952b)

T antigens are present in most strains of group A streptococci and as in the case of the M antigens they are represented by a large number of serologically distinct proteins (see summary in Lancefield 1954) However the distribution of T antigens is not directly related to that of the M antigens In some instances a common or closely related T antigen is found in several different specific M types For example types 15 17 19 23 30 and 47 appear to have closely related T antigens In addition within a single M type certain strains may have one T antigen while others have a totally unrelated T antigen and some strains may have no demonstrable T antigen

The T proteins are resistant to proteolytic digestion and serologically active preparations have been obtained in soluble form by extracting streptococci with proteolytic enzymes Because they are readily destroyed by heat at acid pH ordinarily these antigens are not represented in M-containing extracts Streptococci are agglutinated by antisera containing homologous T antibodies and T antigens have been studied most extensively by this technic Determination of T antigen by agglutination technics can occasionally be a useful adjunct in the identification of streptococci particularly in those cases where

drate is solubilized by the use of cell wall dissolving enzymes, purified preparations also contain muramic acid and amino acids characteristic of the mucopeptide structure found in all bacterial cell walls However the carbohydrate and the mucopeptide are independent elements of the wall and are separable by appropriate chemical technics (Krause and McCarty, 1961) The dominant serologic specificity of the carbohydrate appears to depend on terminal β N acetyl glucosamine residues on side chains or branches (McCarty 1956 1958) These terminal residues can be removed selectively by an induced enzyme obtained from a soil bacillus with concomitant loss of reactivity with group A antisera

In the course of passage of group A strains through animal hosts variants have been isolated which appear to have lost their group specific carbohydrates since extracts do not react with group A antisera (Wilson 1945 Lancefield and Perlmann 1952b) These variants contain a cell wall carbohydrate composed of the same monosaccharides as the group A carbohydrate but with a much higher ratio of rhamnose to N acetyl glucosamine Both the chemical and the serologic differences of the variant carbohydrate are explained by the total absence of terminal N acetyl glucosamine determinants on the side chains The chemical basis for reactivity of variant carbohydrate with homologous antisera is a rhamnose linkage which is also present in group A carbohydrate but is ordinarily masked by the terminal N acetyl glucosamine units (McCarty 1956)

Despite the fact that the group specific carbohydrate occurs near the surface of the cell in the cell wall it does not appear to participate vigorously in agglutination reactions of intact cells However fluorescein labeled antibody to the carbohydrate causes strong specific fluorescence of the entire surface of the cell Agglutination reactions have been shown to be dependent on protein antigens occurring at the cell surface

Soluble preparations of the carbohydrate have little toxicity for animals and are rapidly and quantitatively excreted by the kidney However Schwab *et al* (1959) have shown that cell wall fragments obtained by sonification and composed of the carbohydrate and the mucopeptide elements cause relapsing nodular lesions in the skin of the rabbit.

M Protein *The M proteins are the most* important of the surface proteins They are the antigens which determine the type specificity of group A streptococci and in addition they have been shown to be an important factor in virulence Their role in virulence is comparable with that of the capsular polysaccharides in the virulence of pneumococci That is they serve to inhibit phagocytosis of the organisms by host leukocytes and this antiphagocytic effect is nullified by specific antibody There are more than 40 recognized types of group A streptococci each characterized by a serologically distinct M protein It is indicated both by animal experiments and by observation of natural human infections that protective immunity is directed against the M protein and is therefore type specific The M proteins are firmly attached to the cell surface After mechanical disintegration of streptococci they remain associated with the isolated cell walls Removal of the protein in a soluble and serologically active form from either the intact cell or the cell wall is usually accomplished by the relatively drastic procedure of boiling at pH 2 However enzymatic dissolution of the cell wall in the absence of proteolysis e g with the lytic factor in group C phage lysates releases M protein in a comparable form

The M proteins of the various types of group A streptococci have many properties in common such as their resistance to heat in acid solution and their great susceptibility to proteolytic digestion The susceptibility of M protein to proteolytic enzymes is a property of the native protein and not dependent on previous treatment with acid and heat since it has been shown that this antigen can be destroyed by trypsin treatment of viable streptococci without killing the cells (Lancefield 1943) This fact provides additional evidence for the localization of M protein at the surface of the cell

The typing of group A streptococci is accomplished best by means of a precipitin reaction between an M containing extract of the organism and type specific rabbit antiserum Of necessity this means that there must be available a collection of a large number of different antisera Unfortunately M

TABLE 3 EXTRACELLULAR PRODUCTS OF GROUP A STREPTOCOCCI

	INHIBITION BY SPECIFIC ANTIBODY	DISTRIBUTION	COMMENT
Erythrogenic toxin	+	Produced by majority of strains	Evidence for at least 3 serologically different toxins Production shown to be dependent on infection with temperate bacteriophage in case of Toxin A
Streptolysin S	–	Produced by most strains	Probably cell bound to large extent Released from cell in combination with serum albumin or polyribonucleotides
Streptolysin O	+	Produced by most strains	Active in reduced state only
Diphosphopyridine nucleotidase	+	Production more common among some serologic types than others	
Streptokinase	+	Produced by most strains	
Deoxyribonuclease A	+	One or more produced by all strains	Four serologically distinct enzymes which attack the same substrate
" B	+		
C	+		
D	+		
Hyaluronidase	+	Produced by most members of types 4 & 22 Found only in minute amounts during in vitro growth of other strains	Production enhanced by presence of substrate
Streptococcal proteinase and precursor	+	Produced by majority of strains	Precursor usually found only at pH 5 5 to 6 5 Spontaneous activation under reducing conditions
Amylase	?	Variable but produced by many strains	Production enhanced by presence of substrate
Esterase	–	Produced by most strains	

glycerophosphate isolated from these organisms also react with extracts of most other groups of streptococci as well as those of certain other gram positive bacteria (McCarty 1959) The serologic specificity of this heterophile antigen is dependent on glycerophosphate determinants but in other cases as in the group D and N antigens substituent groups are the primary determinants of specificity The biologic significance of the glycerol teichoic acids remains obscure

L FORMS AND PROTOPLASTS

Typical L forms of group A streptococci were first isolated by Sharp (1954) by anaerobic growth of the organisms on a hypertonic agar medium in the presence of penicillin It was shown by chemical and serologic technics that these L forms lack components characteristic of the cell wall (Sharp *et al* 1957) Following the pioneering work of Weibull on other bacteria, streptococcal protoplasts were later prepared by enzymatic removal of the cell wall in a hypertonic environment that prevented osmotic rupture (Gooder and Maxted 1958 Freimer *et al* 1959) The L forms and the protoplasts are quite similar in their properties and represent forms free of cell wall obtained by two different procedures

Protoplasts of group A streptococci have one unusual property that is not common in the protoplasts of other bacterial species in

little or no M antigen appears to be present However since the T antigens have no known relationship to virulence or protection, identification of this component does not have the same significance as determination of the specific M type

The R antigens represent a third class of surface proteins of group A streptococci which can participate in agglutination reactions The first protein in this category was encountered in type 28 strains and was originally confused with the type specific antigen (Lancefield and Perlmann 1952b) The type 28R antigen resists tryptic digestion but is destroyed by pepsin and it is only slowly destroyed by heat and acid pH Three distinct M types (types 2 28 and 48) have been shown to produce the same R protein (Lancefield 1957) A second R antigen has been found to occur in type 3 strains but so far has not been demonstrated in strains of any other type (Lancefield 1958) The distinction between R and T antigens is not entirely sharp being based primarily on their relative susceptibility to proteolytic digestion and heating in acid and together they form a category of surface proteins which have no detectable relationship to virulence

In view of the complexity of the antigenic structure of group A streptococci it seems probable that there are other as yet unidentified surface antigens However the extensive serologic studies suggest that the M T and R antigens are the most prominent representatives of this class

Hyaluronic Acid Capsule Several independent attempts to demonstrate antigenicity of streptococcal hyaluronate have resulted in failure Despite the apparent nonantigenicity of this substance it is appropriate to consider it with the other surface constituents of the cell because of its peripheral distribution In its native state it is a mucopolysaccharide of high molecular weight which forms highly viscous solutions and is composed of equimolar quantities of N acetyl glucosamine and glucuronic acid It does not appear to be distinguishable chemically from mammalian hyaluronate a fact which may have some bearing on its lack of antigenicity

As indicated in the discussion of morphology of the streptococcal cell in young cultures the hyaluronate surrounds the organism in a well defined capsular structure As growth proceeds the capsules tend to diminish or disappear and the hyaluronate is found in solution in the medium In general the environment provided by host tissues appears to promote capsule formation so that the behavior of capsules in vivo may be quite different from that observed in artificial media

The role of the hyaluronate capsule in virulence is not altogether clear, but there is some evidence that it may help to protect the organism from phagocytosis and destruction and thus potentiate the antiphagocytic effect of the M protein In the case of mouse infections with encapsulated group C streptococci a marked degree of protection can be achieved by treating infected animals with hyaluronidase With group A strains the effect of hyaluronidase treatment of mouse infection is much less dramatic and protection is observed only when very low infecting doses are employed This is in contrast with the marked protective action of type specific anti M serum (Rothbard 1948) The relative importance of hyaluronate and M protein in natural infections of man has not been determined directly although here again it appears probable that the M protein plays a dominant role Studies with in vitro phagocytic systems indicate that normal human serum contains a factor which neutralizes the antiphagocytic effect of hyaluronic acid thus providing an explanation for the limited role of the capsule in human infections (Hirsch and Church 1960, Stollerman 1963)

Glycerol Teichoic Acids As noted above the group-specific antigens of group D and N streptococci belong to the family of bacterial substances designated as glycerol teichoic acids Basically these are polymers of glycero phosphate joined by 1 3 phosphodiester linkages with additional substituents through the hydroxyl at position 2 of the glycerol The most common of these substituents are ester linked D alanine and monosaccharides or oligosaccharides In streptococci these antigens are not bound to the cell wall but nonetheless appear to be present in the surface complex They are much more widely distributed than might appear from their designation as the group specific antigens of groups D and N and rabbit antisera to group A streptococci which react with a poly

TABLE 4 DIFFERENCES IN PROPERTIES OF STREPTOLYSIN O AND S*

	STREPTOLYSIN O	STREPTOLYSIN S
Activation by SH compounds	+	−
Neutralization by specific antibody	+	−
Release enhanced by serum or by polyribonucleotide	−	+
Trypsin sensitivity	+	−
Inhibition by low concentrations of cholesterol	+	−
Inhibition by low concentrations of lecithin	−	+
Rate of lysis	Not directly proportional to lysin concentration	Directly proportional to lysin concentration
Induction period prior to lysis	Short	Long

* Adapted from Bernheimer 1954

of the effects of toxin by antibody is further demonstrated by the fact that the intradermal injection of potent antitoxic sera will result in blanching of the rash in the early stages of scarlet fever (Schulz and Charlton 1918)

The erythrogenic toxins produced by all strains of group A streptococci are not identical Thus Coffey (1938) in a survey of a large number of strains found that the toxins of about 80 per cent were neutralized by a single antitoxic serum while most of the remaining 20 per cent were neutralized by one or the other of two additional antisera The existence of serologically distinct erythrogenic toxins clarifies some of the irregularities observed in reversal of the Dick test after scarlet fever and also provides a rational explanation for the occurrence of second attacks of the disease

Recent evidence (Zabriskie 1964) indicates that the production of erythrogenic toxin by streptococci is associated with the lysogenic state—i e the infection of the organism with certain temperate bacteriophages—in the same way that lysogeny is involved in the production of diphtheria toxin Strains of group A streptococci which do not release detectable toxin can be converted to toxin production by infection with temperate bacteriophages derived from known toxinogenic strains thus providing an explanation for the transmissible toxigenicity first described by Frobisher and Brown (1927) The induction of phage replication and release by ultraviolet irradiation is associated with a sharp increase in the amount of erythrogenic toxin appearing in the culture medium

Preparations of erythrogenic toxin have been obtained in a concentrated and partially purified state with potencies as high as 10^8 skin test doses per μg N (Stock 1939) Progress in the study of the toxin has been limited by the crude nature of the assay procedure which depends on the rather non specific erythematous skin reaction However it is clear that different strains of streptococci vary in the amount of toxin produced in artificial media and the differences are great enough to suggest that they may play a role in the variability of the occurrence of scarlet fever in different epidemics of streptococcal disease Certain strains of group C and group G streptococci have been found to produce a toxin with similar properties

Streptolysin S This agent is responsible for the zones of hemolysis which surround streptococcal colonies on the surface of blood agar plates The designation S refers to serum and derives from the fact that the hemolysin appears to be extracted from living streptococci when they are shaken with serum (Todd 1938) Subsequently Okamoto (1939) found that yeast ribonucleic acid is highly active in inducing release of a hemolysin which appears to be identical with streptolysin S Detailed studies of this material have been made by Okamoto and by Bernheimer (see review by Bernheimer 1954) Recent studies by Ginsburg *et al* (1963) suggest that streptolysin S is in large

that they will proliferate to form colonies in hypertonic agar medium The colonies formed are typical of L-forms, and their growth is unaffected by penicillin in high concentration Despite the absence of the cell wall these forms continue to produce M protein during growth but the antigen is released into the medium rather than being retained on the cell surface In addition, certain of the characteristic extracellular products deoxyribonuclease and a hemolysin can be demonstrated in the agar medium in which protoplasts are grown Adaptation to growth in fluid media has proved to be difficult although it has been achieved with a few strains of L-forms

The cell membrane obtained after osmotic lysis of protoplasts is a lipoprotein structure quite distinct from the cell wall in composition (Freimer 1963) It contains antigens not present in the wall or the cytoplasm and there is preliminary evidence for distinctive antigenic differences between the membranes of different groups of streptococci The finding offers a possible means of identifying L forms isolated from human materials The ability of cell wall free forms of streptococci to survive in vivo and their possible pathogenic significance remain to be explored

Markovitz and Dorfman (1962) have shown that hyaluronic acid can be synthesized from uridine nucleotide intermediates with enzymes present in the protoplast membrane It seems likely the enzymatic systems for the synthesis of other surface components will also be found in the membrane

Extracellular Products of Group A Streptococci

Group A streptococci elaborate a wide variety of biologically active substances including toxins and enzymes which accumulate in the culture medium during growth Not all of these extracellular products are produced simultaneously by any single strain but most strains are capable of forming a majority of them It is known that environmental conditions exert a profound effect on the production of certain of the extracellular substances consequently their occurrence in infected host tissues may differ from that observed in artificial media However since specific antibodies to most of these substances can be found in the sera of patients convalescing from streptococcal infections it is evident that the organisms retain the ability to produce them under in vivo conditions

The list of the known extracellular products (Table 3) probably falls far short of representing the total capacity of group A streptococci in this regard This is indicated not only by the fact that new substances continue to be discovered but also by the results of serologic and electrophoretic studies of concentrated culture fluids which suggest that the known substances can account for only a fraction of the streptococcal protein present

The extent to which the various extracellular substances contribute to the pathogenicity of hemolytic streptococci can for the most part only be surmised since it is difficult to evaluate the importance of a single enzyme or toxin in the over all interaction between parasite and host tissues However on general biologic grounds it seems likely that they are useful to the survival of the organism in its natural habitat which must be assumed to be human tissues The properties of the extracellular substances are discussed in the following sections

Erythrogenic toxin is the substance responsible for the characteristic skin rash of scarlet fever Its mode of action is unknown and the only assay procedure available for the study of the toxin filtrates of streptococcal cultures is a skin test depending on intracutaneous injection of the material in humans (Dick test 1924) or certain susceptible animals The potency of the toxin is such that culture filtrates of appropriate strains can be diluted 100 fold or more and thus the nonspecific irritating effect of other substances in the broth usually can be avoided A positive Dick test is an erythematous and often edematous area of more than 10 mm diameter which appears within 6 to 24 hours

The effects of the toxin are neutralized by antibody A susceptible child shows a positive Dick test and this reactivity persists during the acute phase of scarlet fever However after convalescence the skin test becomes negative in the vast majority of patients as the result of antibody formation Inhibition

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and unrelated technics for inhibiting its bio logic activity oxidation cholesterol inhibi tion and inhibition by specific antibody Thus any property of a streptolysin O preparation which is nullified by all three technics can reasonably be assumed to be due to the hemolysin Using these technics Todd (1942) has presented evidence that streptol ysin O is toxic for leukocytes It has been shown to act as a lethal toxin on intravenous injection into a variety of laboratory animals and one possible explanation for its lethal action has come from studies of its cardio toxicity Bernheimer and Cantoni (1945) first demonstrated that streptolysin O in small amounts causes systolic standstill in the isolated frog heart More recently these studies (Kellner *et al* 1956) have been ex tended to a study of the mammalian heart The isolated hearts of rat rabbit and guinea pig all respond with irreversible loss of myo cardial contractility when as little as 25 to 50 hemolytic units of streptolysin O are added to the perfusion fluid Partially puri fied preparations of the hemolysin have been obtained with a potency as high as 33 000 hemolytic units per mg N and thus it is apparent that very minute amounts are suffi cient to exert a cardiotoxic effect

Halbert *et al* (1961) have extended these studies on cardiotoxicity of streptolysin O to the intact animal and have shown that death from lethal doses is consistently preceded by profound electrocardiographic changes

Diphosphopyridine Nucleotidase (DPN ase)* Streptococcal DPNase was discov- ered in the course of attempts to deter mine the mechanism of the cardiotoxic effect of streptolysin O on mammalian hearts The streptolysin O preparations used were found to inhibit certain of the metabolic enzymes of mammalian heart muscle (Carlson *et al* 1956) but this enzyme inhibition was not affected by any of the procedures known to inactivate the hemolysin Thus it was neces sary to attribute the effect to some unknown component of the preparation The key to its nature was provided by the fact that all of the enzyme systems inhibited involve the co enzyme DPN and direct test revealed the presence of a highly active DPNase which acts by liberating nicotinamide from the molecule (Carlson *et al* 1957)

Streptococcal DPNase is produced by a wide variety of group A strains although many are encountered which do not form appreciable amounts even when grown in the presence of serum a procedure which in creases the yield of the enzyme There appears to be some relationship between DPNase production and serologic type For example Lazarides and Bernheimer (1957) have found that all of 23 strains of type 3 and 52 of 58 strains of type 12 produce the enzyme while all of 33 strains of type 1 and 38 strains of type 19 do not Among other serologic groups certain strains of group C and G possess this property

The enzyme is antigenic and antibodies which inhibit its activity are found in the serum of patients convalescing from strepto coccal infections Ayoub and Wannamaker (1963) have described the occurrence in culture filtrates of a substance possibly an inactive form of the enzyme or a zymogen which binds antibody to DPNase This find ing must be taken into consideration in standardizing methods for the measurement of antibody

A possible toxic role for DPNase has been suggested as a result of studies of the phe nomenon of leukotoxicity This phenomenon first described by Levaditi (1918) years ago is manifested by sudden death and disintegra tion of leukocytes after the ingestion of streptococci Wilson (1957) has shown that certain streptococcal strains consistently exert this effect on a proportion of the leuko cytes which phagocyte them while others apparently lack the ability to injure the leukocyte In a collaborative study (Bern heimer Lazarides and Wilson 1957) involv ing 39 different streptococcal strains it has been found that there is an excellent corre lation between the ability of a strain to pro- duce DPNase and its leukotoxicity All but 2 of the DPNase positive and none of the DPNase negative strains proved to be leuko- toxic While these findings suggest that the enzyme may be concerned in the destruction of the leukocyte some form of direct con firmatory evidence will be needed to establish the relationship

Streptokinase The occurrence of a sub- stance in streptococcal culture filtrates which

* In the new terminology this enzyme is re- ferred to as nicotinamide adenine dinucleotidase (NADase)

part cell bound and that its release from the cell depends on association with some carrier molecule such as serum albumin or ribonucleic acid These observations would explain much of the anomalous behavior of this hemolysin

Partially purified preparations of streptolysin S induced with ribonucleic acid have been obtained with potencies as high as 390 000 hemolytic units per mg N (Bernheimer 1954) These preparations contain both protein and polynucleotide However the activity of the material is destroyed by certain proteolytic enzymes and the hemolytic activity is electrophoretically separable from the polynucleotide, so that it seems probable that streptolysin S is protein in nature Despite this fact the present evidence indicates that streptolysin S is not antigenic or at least that any antibodies formed are not able to neutralize its hemolytic action Inhibitory antibodies are not found in sera of immunized animals or of patients convalescing from streptococcal disease Earlier studies which suggested the occurrence of neutralizing antibody in low titer have been shown to depend on the nonspecific inhibitory effect of serum lipoprotein Phospholipids in the form of lipoprotein complexes appear to play the major role in this inhibition

Attempts to determine whether streptolysin S has toxic effects other than that represented by hemolysis of erythrocytes have been hampered by the fact that pure preparations are not available so it is not possible to be certain that any pharmacologic or pathologic effect observed is not due to some other substance present in the material Evidence for another biologic effect of the hemolysin has been supplied recently by Weismann *et al* (1963) who showed that preparations of both streptolysin S and O cause disruption of lysosomes of mammalian cells and release of lysosomal enzymes

The production of streptolysin S by groups of streptococci other than A has not been studied adequately, although it must be assumed that in most instances an analogous lysin is formed which is responsible for lysis on the surface of blood agar plates No systematic information is available on the effect of ribonucleic acid on the production of hemolysin by other groups

Streptolysin O This hemolysin is so designated because of its oxygen lability and is quite distinct from streptolysin S (see summary of comparative properties in Table 4) It is hemolytically inactive in the oxidized form but is readily activated by the addition of reducing agents such as sulfhydryl compounds This property of reversible oxygen lability is shared with certain other bacterial hemolysins e g , pneumolysin and tetanolysin It is responsible for the failure of streptolysin O to participate in the formation of hemolytic zones around colonies of streptococci grown aerobically on the surface of blood agar The reducing conditions which develop in the environment of deep colonies in blood agar pour plates are sufficient to activate the hemolysin Streptolysin O is produced by almost all strains of group A streptococci and strains which lack this property are encountered only rarely It is also formed by many strains of groups C and G

Streptolysin O has not been obtained in pure form but since it is readily destroyed by proteolytic enzymes it would appear to be protein in nature It is antigenic eliciting the formation of antibodies which effectively neutralize its hemolytic action A high proportion of patients with streptococcal infections show an antibody response during convalescence consequently the measurement of serum antistreptolysin O has become widely used as a test for establishing the occurrence of a recent streptococcal infection Both the oxidized and the reduced forms will combine with specific antibody indicating that reversible oxidation is not associated with major changes in the antigenic properties of streptolysin O Cholesterol is highly active as an inhibitor of streptolysin O For some reason the free cholesterol of normal serum does not exert an appreciable inhibitory effect and does not interfere with the measurement of specific antistreptolysin O However bacterially contaminated serum or serum subjected to certain kinds of chemical treatment may develop inhibitory activity as a result of changes in the lipoproteins

Despite the fact that streptolysin O has not been obtained in pure form study of its biologic effects has met with more success than in the case of streptolysin S This is made possible by the availability of three specific

first serologic studies on deoxyribonuclease (McCarty 1949) has been shown to produce predominantly deoxyribonuclease A and this accounts for the low incidence of antibody responses observed in patients There is little information on the possible diversity of groups C and G deoxyribonucleases although deoxyribonuclease A appears to be the most common enzyme in group C

The production by streptococci of 4 enzymes which attack the same substrate is of theoretic interest It is possible that each enzyme hydrolyzes a different specific linkage in the substrate macromolecule but comparative studies of the split products have not yet been made There is no evidence that deoxyribonuclease attacks living cells and thus it is unlikely that the streptococcal enzymes exert a deleterious effect on tissues during streptococcal infections However they are effective in hydrolyzing the nucleic acids and the nucleoproteins released by necrotic cells and the split products are apparently utilized by the microorganism indicating that the enzymes may have nutritional significance The effect of deoxyribonuclease on nucleoproteins has been exploited as a therapeutic tool in the liquefaction of highly viscous exudates which result from the disintegration of leukocytes as in the case of empyema Preparations containing streptokinase and deoxyribonuclease (streptodornase) from group C strains are commercially available and have been employed in a variety of conditions in which fibrinous or nucleoprotein-containing exudates accumulate (Tillett 1950)

Ribonuclease activity found in culture fluids of group A streptococci (McCarty 1948) is probably due to deoxyribonuclease B (Yasmineh *et al* 1963) Thus this enzyme possesses a rather unusual substrate specificity

Hyaluronidase The production of an extracellular hyaluronidase by an organism which is also able to elaborate a hyaluronic acid capsule would appear at first glance to present something of a paradox especially since it is known that the capsule is destroyed readily by the enzyme However in vitro studies indicate that streptococci rarely produce both substances simultaneously There fore the two functions are to some extent mutually exclusive although there is reason to believe that a single strain may produce capsules while growing under one set of conditions and hyaluronidase under different conditions Hyaluronidase accumulates in relatively large amounts in the culture fluids of most strains of type 4 and type 22 (Crowley 1944) and these strains are apparently never encapsulated Only rare strains of other types have been described as producing comparable amounts of hyaluronidase In the case of the majority of group A strains the presence of an extracellular hyaluronidase can be demonstrated if at all only by highly sensitive tests and often after prolonged incubation even with the use of these technics the amount produced is extremely small compared with that of types 4 and 22 strains The production of the enzyme by types 4 and 22 strains is enhanced by the addition of hyaluronate to the medium (Rogers 1945)

The results of antibody studies suggest that the in vitro findings do not reflect the true potentialities of group A streptococci with respect to hyaluronidase formation Following streptococcal infections a high percentage of patients show an increasing titer of antibodies which specifically inhibit hyaluronidase preparations obtained from types 4 or 22 strains It has been established that this is a true antibody and is not related to the nonspecific hyaluronidase inhibitors found in normal sera Since these antibodies are formed regardless of the type of streptococcus associated with the infection it seems obvious that the pattern of hyaluronidase production observed in laboratory studies is misleading and that most group A strains must produce hyaluronidase during infections in sufficient amounts to stimulate antibody production The explanation of this discrepancy remains obscure and it may involve the action of some undefined factor in the in vivo environment that stimulates production or activation of the enzyme

The understanding of hyaluronidase production by streptococci is further complicated by the finding of Kjems (1958) that this enzyme is also produced during the interaction of many bacteriophages with group A streptococci He believes that these latter enzymes are associated with the bac

promotes the lysis of human fibrin clots was first described by Tillett and Garner (1933) and the active agent was termed streptococcal fibrinolysin Subsequently, it was found (Milstone 1941) that fibrinolysis depends on the presence of an essential serum factor which was later identified as the inactive precursor of a proteolytic enzyme (Christensen 1945) The accumulated evidence indicates that the streptococcal substance exerts its effect by causing the activation of this precursor termed plasminogen and because of the similarity to the activation of trypsinogen by enterokinase the term streptokinase was suggested as more appropriate than fibrinolysin There is a possibility that streptokinase does not act directly on plasminogen but rather on a proactivator which in turn activates plasminogen This human plasma protease system is further complicated by the occurrence of natural inhibitors which affect both the activation process and the enzymatic activity of the protease

Streptokinase is a remarkably potent activator of the human protease system Partially purified preparations containing 2 000 fibrinolytic units per microgram of nitrogen, have been obtained by Christensen (1954) who estimates that as little as 0 00005 mcg of streptokinase nitrogen will exert a detectable effect on a fibrin clot In general it appears to be much less effective in the protease systems of other animal species This may depend on both quantitative and qualitative differences in plasminogen and inhibitors

The biologic activity is readily destroyed by trypsin suggesting that streptokinase is protein in nature It is antigenic and again the antibodies formed specifically inhibit its action The inhibitory effect of antibody can be clearly distinguished from the effects of natural inhibitors of the protease system, and methods have been devised for measuring the amount of antibody in human sera Increases in titer of specific antistreptokinase occur in a high percentage (70 to 80%) of patients following streptococcal infections

Strains of group A streptococci vary over a wide range in the amount of streptokinase produced when the organisms are grown under favorable conditions in artificial media but very few strains appear to lack this property completely Certain group C strains elaborate streptokinase in large amounts, and these have been used in large scale production of the substance which has been undertaken because of the therapeutic value of its fibrinolytic action It is also produced by some group G strains

The possible part that streptokinase plays in the pathogenesis of streptococcal infection is unknown and there is no clear indication that activation of the plasma proteolytic enzyme results in toxic effects or tissue damage However it seems inescapable that streptokinase must prevent the formation of an effective fibrin barrier and thus influence the character of lesions by interfering with localization of the infection

Deoxyribonuclease The supernatant fluid from the growth of certain streptococci can effect rapid depolymerization of deoxyribonucleic acid (Tillett *et al*, 1948, McCarty 1948) This deoxyribonuclease activity is found in cultures of all group A strains in readily detectable amounts and is also present in representatives of several other groups As in the case of many of the other extracellular streptococcal products strains of groups C and G are prominent among the latter

Group A streptococci produce 4 serologically distinct deoxyribonucleases, designated A B C and D (Wannamaker 1958 1962) The enzymes are separable by zone electrophoresis and each is magnesium activated and inhibited by versene Antibodies to the individual enzymes produced in rabbits inhibit the homologous enzyme but do not affect the activity of the others Serum from patients with rheumatic fever or convalescent from streptococcal infections also show marked differences in their ability to inhibit the 4 enzymes A high percentage of patients comparable with that observed in the case of antistreptolysin O produce inhibitory antibody to deoxyribonuclease B while the response to A C and D appears to be inconstant and irregular

Strains of group A streptococci vary in the relative amounts of the 4 enzymes elaborated Some strains produce all 4 enzymes but the predominant enzyme is usually deoxyribonuclease B a fact which is in accord with the serologic findings in patients The organism used in the preparation of enzyme for the

tococcal proteinase causes tissue damage in the course of streptococcal infections The re quirement of low pH and reducing conditions for the release of the enzyme suggests that it will be formed only in certain kinds of infec tion as for example in an abscess where the appropriate environment would be expected That the enzyme is capable of inducing tissue changes in experimental animals has been demonstrated by Kellner and Robertson (1954) who found that intravenous injection of activated streptococcal proteinase results in the formation of necrotic myocardial le sions in rabbits guinea pigs and mice This effect is also shown by other proteolytic en zymes and thus is not specific for strepto coccal proteinase

Amylase Many strains of group A strepto cocci elaborate an extracellular amylase when grown in the customary peptone media (Crowley 1950 1954) The enzyme has the properties of an α amylase and hydrolyzes glycogen and amylopectin in addition to starch Production of amylase varies greatly among strains and even individual members of a population of a single strain may show differences in enzyme activity The presence of substrate in the medium greatly enhances the amount of amylase released

Further studies have revealed that the same strains which produce amylase will under different cultural conditions synthe size a starchlike polysaccharide (Crowley 1955 Crowley and Jevons 1955) The presence of human plasma in the medium is important for the formation of the sub stance and maltose serves as an adequate sub strate although starch and glycogen are also effective These findings provide another ex ample of the marked influence of environ mental conditions on the biologic behavior of streptococci

The antigenicity of amylase and the pos sible occurrence of antibodies in human sera have not been investigated

Esterase Stock *et al* (1961) have de scribed the production by group A strepto cocci of an extracellular esterase which acts on the substrate β naphthyl acetate The antibody to this enzyme found in rabbit and human antisera does not inhibit the action of the enzyme

Intracellular Components

The intracellular components of group A streptococci have received little attention in comparison with that given to the surface constituents and extracellular products In part this is referable to the fact that cellular extracts represent highly complex mixtures which are difficult to resolve Such extracts contain a variety of proteins including the various metabolic and synthetic enzymes of the cell and large amounts of other material such as nucleotides and nucleic acids A nucleoprotein fraction obtained by extrac tion with weak alkali has been examined for its serologic properties (Lancefield 1925) Although this material probably makes up a large portion of the total cellular contents and must be very heterogeneous antigenically the serologic studies are useful in indicating interrelationships between bacterial species Thus nucleoproteins from hemolytic strepto cocci react with antisera from animals im munized with nucleoproteins from both hemolytic and nonhemolytic streptococci as well as with similar preparations obtained from pneumococci A lesser degree of cross reactivity was observed with antisera to staphylococcal nucleoproteins

The properties of individual cellular en zymes have been investigated in only a few instances Jacox (1953) reported the occur rence of a β glucuronidase which he found in representatives of only 4 of 32 different types of group A streptococci There was no evi dence of inhibition of the enzyme by the sera of patients convalescing from streptococcal infections The lipoproteinase described by Krumwiede (1954) which causes opal escence in serum by splitting α_1 lipoprotein probably represents a cellular enzyme since it is extractable from washed organisms with 40 per cent urea However it may be re leased into the environment under conditions reminiscent of those which affect the produc tion of streptolysin S that is it is found in serum broth cultures and appears to be ex tractable from cells with serum Preliminary studies suggest that certain human sera may contain antibodies which inhibit lipopro teinase and inhibitory antisera have been ob tained by immunizing rabbits with crude enzyme preparations

teriophage and are serologically distinct from the streptococcal hyaluronidase The relationship is therefore somewhat different from that observed in the case of erythrogenic toxin

Streptococcal hyaluronidase differs from testicular hyaluronidase in several respects The end products of hydrolysis by the bacterial enzyme are not identical with those of the mammalian enzyme and the former does not attack chondroitin sulfate

Hyaluronidase production occurs in streptococci other than those of group A notably in certain strains of groups B, C and G In the case of groups C and G it has been shown that the enzymes are serologically distinct from those of group A and are not significantly inhibited by antibody to the group A enzyme

Streptococcal Proteinase This enzyme and its inactive precursor are the only extracellular substances which have been obtained in crystalline form (Elliott 1950) They are also unique by virtue of the marked effect exerted by environmental conditions on their production Proteinase precursor is released by most strains of streptococci only when the pH of the medium is maintained between 5 5 and 6 5 Thus except in the case of certain unusual strains none of this substance is found under the optimal conditions for growth of the organisms between pH 7 0 and pH 8 0 which also favor the production of other extracellular enzymes As in the case of streptolysin S and deoxyribonuclease precursor is formed by washed streptococcal cells when suspended in an incomplete medium Salts and glucose are sufficient for this purpose if the pH is maintained below 6 5 but production is greatly enhanced by the addition of peptone products The capacity of streptococci to produce this substance under appropriate conditions appears to be much greater than for the other extracellular enzymes and Elliott (1950) reports the recovery of 169 mg of 6 times recrystallized material from 10 liters of culture filtrate

The precursor is autocatalytically converted to active proteinase under suitable reducing conditions (Elliott and Dole 1947) This conversion may occur in culture media if aerobic conditions do not prevent a fall in oxidation reduction potential and can be achieved in solutions of the crystalline precursor by the addition of sulfhydryl compounds or other reducing agents The autocatalytic effect appears to depend on the initial presence of traces of active enzyme The action of trypsin in low concentration also brings about rapid conversion of the precursor Formation of active enzyme by either method is accompanied by the release of dialyzable split products amounting to 40 per cent of the weight of crystalline precursor Streptococcal proteinase resembles cathepsins and plant enzymes of the papain family in requiring activation by sulfhydryl compounds or comparable reducing agents Studies on synthetic substrates indicate that it has an unusually broad substrate specificity (Myceк *et al* 1952)

The production of proteinase by streptococci appears to be largely limited to members of group A It has been found in representative cultures of most of the serologic types although enhancement of virulence by mouse passage results in suppression of its production (Elliott 1945) Growth of streptococci under conditions which allow the appearance of active proteinase in the medium can have a profound effect on other biologic properties Thus the M protein is destroyed because of its great susceptibility to proteolytic digestion and serologic typing of the organisms becomes impossible (Elliott 1945) Furthermore certain of the other extracellular products e g streptokinase and hyaluronidase may be destroyed by proteolytic action

Precipitating antibodies have been prepared in rabbits against both crystalline proteins The proteins differ serologically and the precursor behaves as though it has 2 antigenic components one of which is specific and the other identical with the proteinase antigen (Elliott 1950) Rabbit and horse antibodies to proteinase have been shown to inhibit the action of the enzyme However inhibitory antibodies are not readily demonstrated in sera of patients after streptococcal infections but it has been possible by the use of a hemagglutination technic to demonstrate that antibodies are produced by a large proportion of such patients (Ogburn *et al* 1958) It is not known whether or not strep

partum endometrium and septicemia of the newborn can be caused by these organisms

Hemolytic streptococcal infections are to be distinguished from infections associated with the various nonhemolytic streptococci which are found among the normal flora of the respiratory and the gastrointestinal tracts These organisms frequently find their way into the bloodstream in small numbers but under ordinary conditions they are readily disposed of by the natural defense mechanisms However in the presence of anomalies of anatomic structure they may become established as infectious agents Thus subacute bacterial endocarditis which affects heart valves altered by congenital malformations or rheumatic heart disease is most frequently associated with this class of organisms Similarly infections of the urinary tract with nonhemolytic streptococci may be encountered when anomalies or obstruction of the outflow tract are present The nature of the organism which lodges on the heart valves in endocarditis would appear to be a matter of chance rather than the expression of specific pathogenic properties Although the streptococci involved usually do not belong to one of the recognized groups members of certain serologic groups are also encountered For example group D streptococci which normally inhabit the gastrointestinal tract (enterococci) are often incriminated in endocarditis as well as in urinary tract infection Members of other groups (e g group O) are also occasionally isolated from the bloodstream of patients with endocarditis

Because of the pre eminent importance of group A streptococci in human disease the following sections deal primarily with the characteristics of infections with these organisms

Respiratory Tract

The primary site of invasion of the human body by group A streptococci is through the upper respiratory tract Streptococcal pharyngitis or tonsillitis is by far the most common of all streptococcal infections and provides the focus from which the organisms are disseminated to initiate most other forms of streptococcal disease

Streptococci appear to have a special predilection for the lymphatic system and in the upper respiratory tract the initial localization of the infection is in the lymphoid tissue of the pharynx The infectious process is manifested by swelling and reddening of the tonsils and other pharyngeal lymphoid tissue and in the classic disease is accompanied by the appearance of focal or confluent accumulations of exudate on the affected areas The adjacent mucous membranes are also commonly involved in the inflammatory process These changes cause soreness of the throat which may be quite marked and in addition the infection leads to systemic manifestations such as fever and general toxicity While this represents the typical picture of the full blown disease streptococcal pharyngitis can also occur with only minimal evidence of inflammation and minimal symptoms

Complications of this relatively superficial infection occur by extension either through lymphatic channels or by direct involvement of other areas The cervical lymph nodes at the angle of the jaw which drain the tonsillar area are usually involved to some degree and become swollen and tender In severe cases infection of these nodes may progress to the formation of purulent abscesses Abscesses may also form in the deep peritonsillar tissues (quinsy) and particularly in infants in the retropharyngeal tissues Like many of the other severe manifestations of streptococcal disease peritonsillar and retropharyngeal abscesses are now relatively rare This is true also of Ludwig s angina which represents cellulitis and subsequent abscess formation in the tissues of the neck especially at the base of the tongue and on the floor of the mouth The extensive swelling associated with all three of these conditions often leads to difficulty in swallowing and serious embarrassment of respiration

Streptococcal infections of the pharynx readily extend to the paranasal sinuses through the natural openings Similarly the eustachian tube is frequently involved and leads to acute infections of the middle ear Both the sinusitis and the otitis media which result from these extensions may develop into chronic purulent processes In the case of otitis media the infection can invade the air cells of the mastoid bone causing mas

Attempts have been made to resolve the cellular components of group A streptococci into definable fractions by electrophoretic technics (Hess and Slade 1955) These experiments involve extracts obtained after disintegration of the cells by sonic oscillation or shaking with glass beads The electrophoretic patterns obtained with extracts of various types have been compared but there is still little information on the biologic activity of the several electrophoretic components

RELATIONSHIP BETWEEN STREPTOCOCCI OF GROUP A AND OTHER GROUPS

The basic similarity in the monosaccharide composition of the cell wall carbohydrates of groups A through G has already been mentioned There is in addition evidence for a close biologic relationship between groups A C and G which is based largely on the study of strains of human origin For example it is evident from the discussion of the extracellular products of group A streptococci that the same or similar substances are in most cases also produced by some strains of groups C and G Certain of the enzymes such as streptokinase and deoxyribonuclease may be found in cultures of some other groups but it is only in groups C and G that one finds a total complement of extracellular substances approaching that of group A Further support for the interrelationship comes from studies on the distribution of surface protein antigens Thus R antigen serologically identical or closely related to the group A substance has been found in members of groups C and G streptococci as well as in some of group B (Maxted 1949)

The group specific carbohydrate of group C appears to differ from that of group A primarily in the fact that N acetyl galactosamine rather than N acetyl glucosamine has a terminal position on the side-chains and serves as the major antigenic determinant (Krause and McCarty 1962) The structure of the remainder of the molecule is essentially the same and mutants of group C streptococci have been isolated which produce a carbohydrate lacking the N acetyl galactosamine determinants and possessing the chemical and serologic properties of group A variant carbohydrate (Araujo and Krause, 1963)

Several of the streptococcal groups other than A have been subdivided into serologic types but in no case has a type specific protein antigen been identified which has been clearly shown to have a relationship to virulence comparable with that of M protein Type differentiation in group C streptococci has not been explored intensively, but it appears to depend on surface protein antigens and it is possible that in some instances these may be M like in their biologic properties In the case of other groups in which specific types have been described (groups B D, F and G) the available evidence suggests that the type specific substance is polysaccharide in nature This has been clearly established in the case of group B streptococci which have polysaccharide capsules analogous to those of pneumococci

The various similarities and dissimilarities between hemolytic streptococci do not fall in sufficiently uniform patterns to allow for many broad generalizations On the whole however it seems clear that they are much more closely related to one another than to the numerous nonhemolytic and anaerobic streptococci

HEMOLYTIC STREPTOCOCCAL INFECTIONS IN MAN

Hemolytic streptococci are associated with a wide variety of disease entities in man and in the vast majority of these acute infections the causative organisms belong to group A Streptococci of groups C and G, possibly as a reflection of their biologic similarities to group A are sometimes implicated in similar infections especially those involving the upper respiratory tract These infections with groups C and G streptococci are characteristically mild and usually do not lead to the complications observed in group A infections They are not known to initiate late sequelae such as rheumatic fever or glomerulonephritis Occasionally other groups of streptococci are encountered in association with pathologic processes which are more commonly caused by group A streptococci For example puerperal fever can result from group B streptococcal infection of the post

more commonly associated with scarlet fever than others This may conceivably be refer able to the relative capacity of individual strains to produce erythrogenic toxin in large amounts in vivo but a correlation of this kind has not been demonstrated by labora tory studies

Scarlet fever has been described in asso- ciation with upper respiratory infections with group C streptococci and even in infections with more distantly related organisms the staphylococci In the latter case the staphylo cocci involved are found to produce an erythrogenic toxin with properties like that of the streptococcal toxin However these are relatively rare exceptions and in nearly all cases of the disease group A streptococci are recovered on culture

OTHER ACUTE STREPTOCOCCAL INFECTIONS

While the upper respiratory tract is the principal site of group A streptococcal infec tions these organisms have the capacity to localize and produce infections in many other areas The initiation of infection in these areas may result either from hematogenous spread or from the chance inoculation of susceptible tissues with organisms from the environment The adaptability of streptococci to the in vasion of human tissues is well illustrated by their behavior when introduced directly through the normal skin barrier This can occur in a variety of accidental injuries as in the case of infection of cuts or blisters or in natural portals of entry such as the umbilical cord of the newborn infant The course of events following production of le sions of this type by virulent streptococci in the extremities follows a well defined pattern *The lymphatic vessels are quickly involved* often with minimal evidence of reaction at the site of the local lesion and the occur rence of advancing lymphangitis is visibly manifested by the appearance of red streaks moving up the arm or the leg The draining lymph nodes become swollen and tender but do not significantly check the centripetal spread of the lymphatic infection which con tinues until it reaches the bloodstream and gives rise to septicemia Frequently the progress of this type of infection is so rapid that septicemia and death can occur within 24 to 48 hours of the initial injury Varia tions in virulence of the organism or in host resistance may alter this course of events and one may even encounter localized purulent infections of the skin which spread only in an indolent fashion

Septicemia may develop as a complication of other localized streptococcal infections It is always a most serious manifestation ac companied by high fever and severe toxic symptoms Prior to the discovery of effective chemotherapeutic agents the occurrence of septicemia was always a grave prognostic sign In contrast with this form of blood stream infection which is always associated with some focus that provides a continuing supply of organisms transient bacteremia can occur without giving rise to apparent symp tomatology unless the organisms are able to establish themselves at another site This phenomenon is responsible for hematogenous dissemination of streptococcal infection Thus septic arthritis manifested by the ac cumulation of purulent infected exudate within the joint cavity must be assumed to originate by this process Similarly strepto coccal meningitis sometimes appears in the absence of demonstrable mastoiditis suggest ing that it originates by way of the blood stream rather than by direct contiguity

The classic puerperal fever in its most viru lent form represents a group A streptococcal infection of the postpartum endometrium with subsequent septicemia The evidence in dicates that this endometritis results from direct infection of a highly susceptible tissue At the time that this infection was widely prevalent it was noted that its greatest inci dence coincided with epidemics of scarlet fever and streptococcal sore throat increas *ing the opportunity for dissemination of* streptococci from the respiratory tracts of carriers who came into contact with the pa tient The institution of suitable aseptic tech nics did much to reduce the danger of puer peral fever even as it reduced the occurrence of streptococcal infection of surgical wounds

Erysipelas is a streptococcal skin infection which like scarlet fever was originally thought to be a specific disease until it was demonstrated that the organisms responsible belong to the same serologic types as those causing other streptococcal infections This infection characteristically spreads in all di

toiditis and ultimately osteomyelitis of the surrounding bony structures If invasion of the bone progresses to the meningeal surfaces serious complications such as meningitis or cerebral sinus thrombosis may result

Extension of streptococcal pharyngitis may also occur downward into the lower respiratory tract Here it gives rise to bronchitis and interstitial bronchopneumonia characterized by extensive involvement of the lymphatic vessels Spread of the infection by the lymphatics typical of the streptococcal lymphangitis in other areas follows the flow to the draining lymph nodes but may proceed in a retrograde direction in occluded vessels and reach the pleural surfaces Because of this retrograde extension pleurisy is common in streptococcal pneumonia and leads to the formation of large quantities of serofibrinous pleural exudate

Relative to the great frequency of upper respiratory infections with streptococci pneumonia is a rare event Certain viral infections of the respiratory tract seem to promote the occurrence of this complication when they occur simultaneously with streptococcal infection During World War I for example a large number of cases of streptococcal pneumonia with massive pleural effusion were encountered among military personnel in the course of measles and influenza epidemics

Scarlet Fever

Scarlet fever was recognized as one of the common exanthematous diseases of children for centuries before its streptococcal etiology was discovered Because of this history it is usually considered as a separate disease entity although modern bacteriologic findings indicate that it merely represents streptococcal pharyngitis or tonsillitis with an accompanying rash Thus in a given epidemic of streptococcal disease a single predominant type of group A streptococcus may be recovered from cases of both scarlet fever and streptococcal sore throat

The erythrogenic toxin is responsible for the rash of scarlet fever but the importance of this substance in other toxic manifestations of the disease has not been clearly established As indicated in the discussion of the extracellular products of group A streptococci this substance is only one of several with potentially toxic properties Symptoms of systemic toxicity occur in the case of streptococcal sore throat which are indistinguishable from those of scarlet fever and the degree of this manifestation is more a function of the severity of the disease process than of the presence or the absence of a skin rash Other clinical findings of scarlet fever such as the so called strawberry tongue resulting from the appearance of prominent papillae on an otherwise smooth and diffusely reddened organ also occur in uncomplicated streptococcal pharyngitis The purulent complications of scarlet fever (cervical adenitis sinusitis otitis etc) are identical with those described for streptococcal pharyngitis and the same delayed nonsuppurative sequelae are encountered

The rash of scarlet fever is characterized by diffuse reddening of the skin which may cover most of the body but is frequently most prominent on the trunk The changes induced in the skin result in desquamation during convalescence This is especially evident on the hands and the feet where a thick keratinized layer is present

There are certain anomalies in the occurrence of scarlet fever which are not satisfactorily explained on the basis of susceptibility or immunity to a primary toxin For example, the disease is rarely seen in infants and very young children and infants do not give positive reactions with Dick toxin These findings cannot be correlated with the transfer of passive immunity from the mother and have led to the suggestion that the development of hypersensitivity through prior exposure to erythrogenic toxin is required before this agent can induce the formation of the characteristic rash In any event it is clear that immunity to the toxin is effective in preventing second attacks of the scarlet fever in the vast majority of cases, although it has no effect on the occurrence of subsequent streptococcal infections As noted earlier the occasional occurrence of second attacks of scarlet fever can be explained by the fact that certain strains of group A streptococci produce an erythrogenic toxin which is serologically distinct from that of the majority of strains It is evident also that some epidemics of streptococcal disease are much

majority of recovered patients do not show evidence of chronic kidney damage Further more recurrent attacks of glomerulonephritis are rare

An additional important difference in the pathogenesis of the two diseases has been established recently by the finding that only certain types of group A streptococci appear to be associated with the initiation of glomerulonephritis (Rammelkamp and Weaver 1953) These studies now adequately confirmed show that the great majority of cases are preceded by type 12 streptococcal infections and that the remaining cases are usually attributable to one or two additional types This strongly suggests that some specific property of certain strains of group A streptococci is important in the etiology of glomerulonephritis These findings also serve to explain the rarity of second attacks of the disease as contrasted with the frequent recurrence of rheumatic fever

Erythema nodosum the third representative of this group of diseases is less clearly delineated and only a portion of the cases showing the clinical picture of the disease can be attributed to a preceding streptococcal infection However when this relationship exists it follows the same pattern of delayed appearance after the bacterial infection and can recur following subsequent attacks of streptococcal sore throat The disease is manifested by the occurrence of red extremely tender nodular swellings particularly on the extensor surfaces of the extremities in association with fever and signs of general toxicity Lesions of the same type may be encountered occasionally in rheumatic fever but erythema nodosum is seen more commonly in the absence of other rheumatic stigmata and recovery occurs without residual damage

There is evidence to suggest that these late sequelae of streptococcal infection may represent a severe and exaggerated form of a type of host reaction that is commonly manifested during convalescence Thus during the period following apparent recovery from the acute infections one may encounter recurrent swelling and tenderness of the cervical lymph nodes transient arthralgias transient electrocardiographic changes microscopic hematuria etc The time of appearance of these changes as well as the time of onset of the overt poststreptococcal diseases coincides with the time at which antibody response to streptococcal antigens is reaching its maximum and this has been an important factor in the formulation of the theory that hyper sensitivity reactions are concerned with pathogenesis of the delayed sequelae

IMMUNITY

As noted earlier specific antibodies to most of the extracellular products of group A streptococci are demonstrable in the serum of a large percentage of patients after recovery from streptococcal infections Similarly certain of the surface antigens and cellular components have been shown to induce antibody response (see review in McCarty 1954) Furthermore even though the number of individual streptococcal antigens which have been studied is relatively large it is evident that this represents only a portion of the potential antigens These findings emphasize perhaps more clearly than in the case of any other bacterial infection that the patient with a streptococcal infection is exposed to a wide variety of different antigenic substances during the active disease However there are few instances in which the antibodies produced can be related to immunity to infection Among the several extracellular substances for example only the antibody to the erythrogenic toxin has been shown to have a readily demonstrable effect on a disease manifestation Since the antibodies to other toxins and enzymes usually inhibit their action it is conceivable that they may affect the development of the disease process in a manner that remains undefined

In general immunity to streptococcal infection is type-specific and depends on the production of antibodies to M protein This has been demonstrated both in experimental infections in laboratory animals and in natural infections in man Studies of repeated attacks of streptococcal pharyngitis in a single individual have shown that each episode is associated with a different type of group A streptococcus and that recurrent infections with the same type are extremely rare In this connection it should be noted that effective penicillin therapy can suppress the antibody

rections in the subepidermal tissues from an original focus which may or may not be readily apparent The inflammatory process is manifested by redness and edema and the *advancing elevated margin of the lesion is* clearly demarcated from normal skin Streptococci are usually demonstrable only in the edema fluid from the advancing edge The area of skin involved may be extensive and in severe cases deeper penetration or septicemia can ensue

Streptococci are also involved in the superficial purulent skin lesions of impetigo contagiosa Here they are usually found in association with staphylococci and the lesions assume a *more chronic and indolent* character They begin as individual vesicles which subsequently break with the formation of encrusted purulent lesions

Delayed Sequelae of Streptococcal Infections

This group of diseases includes acute rheumatic fever acute hemorrhagic glomerulonephritis and erythema nodosum They represent an entirely different order of disease phenomena from the various suppurative complications of acute streptococcal disease in which the infecting organisms play so prominent a role The mechanisms involved in the initiation of the delayed sequelae by streptococcal infection have not been clarified but it is evident not only that the clinical findings are unique but also that the nature of the pathologic lesions is distinct from that of the processes in which direct bacterial involvement is evident

Rheumatic fever is the most important and *widespread of the late sequelae* Characteristically this disease is preceded by a typical streptococcal infection of the upper respiratory tract and has its onset after a latent interval which is variable in length with an average of about 3 weeks The nature and the severity of the *symptoms of rheumatic fever* cover a wide range However the most prominent features of the classic disease are fever, migratory polyarthritis and carditis The arthritis is not to be confused with the acute septic arthritis mentioned previously since streptococci are *not demonstrable in* the joint fluid and recovery occurs without residual damage to the joint Involvement of the heart is the most significant aspect of the disease from the point of view of both the severity of the acute illness and the production of permanent damage All layers of the *heart may be affected* and signs of myocarditis and pericarditis are frequently present The basic pathologic finding is a focal inflammatory process characterized by the occurrence of unique myocardial lesions known as Aschoff bodies Involvement of the endocardium can result in deformative scarring of the valves and adjacent structures which leads to chronic rheumatic valvular heart disease

The generalized nature of the rheumatic *process is illustrated* by the variety of other manifestations which may occur in the course of the disease including involvement of the skin and of the central nervous system (chorea) The disease may become subacute or chronic and in extreme cases evidence of rheumatic activity may persist *for years After* recovery from rheumatic fever the patient is highly susceptible to recurrence of the disease following a new streptococcal infection and many attacks can occur in a single individual

There appears to be no strain selectivity among group A streptococci in their ability to initiate rheumatic fever since practically all of the prevalent serologic types have been found to be associated with the disease However only a small percentage of patients with streptococcal sore throat develop this late complication and it is evident that host factors must play a role in the pathogenesis of the disease

Acute hemorrhagic glomerulonephritis re*sembles rheumatic* fever in the occurrence of a latent interval between the streptococcal infection and onset of the disease although in this case the average length of the interval is probably shorter As the name suggests this disease involves primarily the glomerulus *of the kidney and is associated* with the appearance of blood in the urine and general manifestations of kidney disease such as edema and hypertension As might be expected from their common origin rheumatic fever and glomerulonephritis occasionally *occur simultaneously in the same* patient However there is less evidence of permanent damage in glomerulonephritis and the great

majority of recovered patients do not show evidence of chronic kidney damage Furthermore recurrent attacks of glomerulonephritis are rare

An *additional important difference* in the pathogenesis of the two diseases has been established recently by the finding that only certain types of group A streptococci appear to be associated with the initiation of glomerulonephritis (Rammelkamp and Weaver 1953) These studies now adequately confirmed show that the great majority of cases are preceded by type 12 streptococcal infections and that the remaining cases are usually attributable to one or two additional types This strongly suggests that some specific property of certain strains of group A streptococci is important in the etiology of glomerulonephritis These findings also serve to explain the rarity of second attacks of the disease as contrasted with the frequent recurrence of rheumatic fever

Erythema nodosum the third representative of this group of diseases is less clearly delineated and only a portion of the cases showing the clinical picture of the disease can be attributed to a preceding streptococcal infection However when this relationship exists it follows the same pattern of delayed appearance after the bacterial infection and can recur following subsequent attacks of streptococcal sore throat The disease is manifested by the occurrence of red extremely tender nodular swellings particularly on the extensor surfaces of the extremities in association with fever and signs of general toxicity Lesions of the same type may be encountered occasionally in rheumatic fever but erythema nodosum is seen more commonly in the absence of other rheumatic stigmata and recovery occurs without residual damage

There is evidence to suggest that these late sequelae of streptococcal infection may represent a severe and exaggerated form of a type of host reaction that is commonly manifested during convalescence Thus during the period following apparent recovery from the acute infections one may encounter recurrent swelling and tenderness of the cervical lymph nodes transient arthralgias transient electrocardiographic changes microscopic hematuria etc The time of appearance of these changes as well as the time of onset of the overt poststreptococcal diseases coincides with the time at which antibody response to streptococcal antigens is reaching its maximum and this has been an important factor in the formulation of the theory that hypersensitivity reactions are concerned with pathogenesis of the delayed sequelae

IMMUNITY

As noted earlier specific antibodies to most of the extracellular products of group A streptococci are demonstrable in the serum of a large percentage of patients after recovery from streptococcal infections Similarly certain of the surface antigens and cellular components have been shown to induce antibody response (see review in McCarty 1954) Furthermore even though the number of individual streptococcal antigens which have been studied is relatively large it is evident that this represents only a portion of the potential antigens These findings emphasize perhaps more clearly than in the case of any other bacterial infection that the patient with a streptococcal infection is exposed to a wide variety of different antigenic substances during the active disease However there are few instances in which the antibodies produced can be related to immunity to infection Among the several extracellular substances for example only the antibody to the erythrogenic toxin has been shown to have a readily demonstrable effect on a disease manifestation Since the antibodies to other toxins and enzymes usually inhibit their action it is conceivable that they may affect the development of the disease process in a manner that remains undefined

In general immunity to streptococcal infection is type-specific and depends on the production of antibodies to M protein This has been demonstrated both in experimental infections in laboratory animals and in natural infections in man Studies of repeated attacks of streptococcal pharyngitis in a single individual have shown that each episode is associated with a different type of group A streptococcus and that recurrent infections with the same type are extremely rare In this connection it should be noted that effective penicillin therapy can suppress the antibody

response to various streptococcal antigens, including the extracellular products and M protein, and consequently may result in an increased incidence of second infections with a single type Following untreated infection, the great majority of patients develop M antibody which is measurable by the bactericidal technic previously described There is evidence that type specific antibody may persist in detectable amounts for many years and thus provide a basis for prolonged immunity to a given type

Although type specific immunity has been established as a major factor in streptococcal disease certain aspects of the natural history of these infections suggest the possibility of some form of nonspecific resistance or immunity Experiments with laboratory animals have contributed little in this regard In the naturally occurring disease streptococcal infections are most common among school children and there is a progressive decrease in incidence with advancing age Considering the large number of types of group A streptococci which may be prevalent at different times it is difficult to ascribe the relative immunity of adults entirely to type specific immunity However, many factors other than humoral immunity may play a role in this problem and under appropriate conditions of increased exposure as in military establishments the incidence of streptococcal infections in adults can be very high

In addition to a decrease in incidence of streptococcal infections with age there are changes in the characteristic manifestations of the disease that may be related to the immune response For example in infants under 3 years of age the disease is commonly a low grade subacute process which can last several weeks and often does not show the clear cut localizing signs that characterize streptococcal pharyngitis in the older child and adult These changes have been emphasized by Powers and Boisvert (1944) who suggested the term streptococcosis for all streptococcal disease in analogy with the course of events in tuberculosis The development of immunity and hypersensitivity is considered to play a major part in the changing patterns of both diseases There is evidence that the vigorous type of antibody response to a variety of streptococcal antigens in children and adults may be dependent on prior conditioning during streptococcal infections in infancy Thus, it has been found (Rantz *et al*, 1951) that the production of antistreptolysin O is often very feeble in young infants and that even in children from 1 to 6 years of age increased antibody levels are maintained for a shorter period of time than in older children In addition, the appearance of detectable levels of M antibody after infection is usually greatly delayed in comparison with antibodies to the extracellular products This can be explained best by assuming that the latter represent the secondary or anamnestic type of antibody response while the M protein is a new antigen to which the individual has not been exposed previously If this explanation is correct it supports the view that the antistreptolysin O type of response characteristic of the older patient is conditioned by prior exposure to the antigen

During the past several decades there seems to have been a progressive decline in the severity of streptococcal infections This is most evident in the case of readily recognizable manifestations such as scarlet fever This decrease in severity unaccompanied by a comparable decrease in the morbidity rate antedates the introduction of *antibacterial* drugs and it must be assumed that either changes in the virulence of the organism or in the susceptibility of the human population or possibly a combination of both factors are responsible Similar changes in the severity of scarlet fever were reported in past centuries and although the validity of these older observations is uncertain the possibility of recurring cycles of varying disease intensity is suggested The fact that the immunity status of a population can play a key role in the severity of streptococcal disease is indicated by the highly fatal streptococcal epidemics that are seen occasionally in isolated groups not previously exposed to streptococci It is evident that the matter of nonspecific immunity to streptococcal infection requires further study

Immunity in rheumatic fever is no more than a special case of streptococcal *immunity* The same antibodies are formed as in streptococcal infections which do not give rise to this delayed complication However, there

may be a quantitative difference since in a given epidemic of streptococcal disease the mean antibody response of rheumatic fever patients to a variety of streptococcal antigens is greater than in patients with uncomplicated infections In the case of glomerulonephritis the importance of type specific immunity is emphasized As pointed out earlier the low incidence of recurrences of this disease in contrast with rheumatic fever probably depends on the fact that a single type of group A streptococcus is responsible for most of the disease Thus the development of M antibody to this type during the first attack greatly reduces the chance of a recurrence

DIAGNOSIS

Few of the many manifestations of streptococcal disease are sufficiently distinctive to allow for conclusive diagnosis on clinical grounds alone Scarlet fever and erysipelas exemplify the more readily recognizable streptococcal infections and classic attacks can be diagnosed with reasonable accuracy from the signs and symptoms However the findings in streptococcal pharyngitis and tonsillitis are much more variable than they were once thought to be In many cases exudate is absent and the inflammation of the pharynx cannot be distinguished from that caused by a variety of other upper respiratory infections Even when exudate or follicular tonsillitis is present the diagnosis is not certain because of the occurrence of non streptococcal infections which give a similar picture It must be concluded that isolation of the organism by bacteriologic culture is of primary importance in accurate diagnosis

The material for culture is most commonly obtained from the throat or the nasopharynx by the use of a sterile swab It is important to transfer the material promptly to the culture medium without allowing the swab to become dry This is done most conveniently by rolling the swab on the surface of a blood agar plate after which the inoculum is streaked out with a sterile bacteriologic loop In this way an adequate distribution of the material is usually obtained so that isolated colonies appear The nature of the blood used is of importance and sheep blood is recommended because of its property of suppressing the growth of certain hemolytic members of the genus Hemophilus However the zones of hemolysis are not always typical and some workers use both rabbit and sheep blood plates in the initial isolation of streptococci The use of blood of other species (human horse) may lead to difficulties in the recognition of hemolytic colonies and requires special care and experience In order to obtain typical colony formation it is preferable to use fresh blood agar plates or plates that have been sealed to prevent the loss of moisture during refrigeration

After overnight incubation the plates are examined for the presence of hemolytic colonies In the majority of streptococcal infections a large number of characteristic colonies with distinct hemolytic zones will be apparent if a satisfactory culture technic has been employed In some instances however because of the occurrence of strains which show little hemolysis on initial isolation or do not produce streptolysin S the presence of streptococci may not be obvious Experience is required to recognize colonies of these strains although certain technics may assist in identifying them Thus when streaking the culture one may make a stab deep into the agar through the heavily inoculated portion of the plate so that subsurface colonies will form which can show hemolysis as the result of production of streptolysin O Some workers recommend the use of anaerobic culture conditions as a means of increasing the efficiency of isolating streptococci on initial culture

In view of the primary importance of group A streptococci and the possible occurrence of streptococci of other groups in the human *throat further identification of the organism* should be carried out by serologic grouping Since grouping antisera are now available commercially this additional step is feasible in most diagnostic laboratories For this purpose isolation of individual colonies and growth in pure culture in amounts sufficient to prepare extracts containing the group-specific carbohydrate are required Typing of proved group A streptococci has a more limited application and usually is carried out only in connection with special studies

Some confusion may result in attempting to differentiate streptococcal carriers from

patients with acute streptococcal disease by means of cultural studies However, group A streptococci are not often the predominant organism in the throat of carriers, and whenever these organisms are present in large numbers in association with symptoms of respiratory disease the diagnosis of streptococcal infection must be made

The use of cultures is of relatively little value in the diagnosis of rheumatic fever and glomerulonephritis since the initiating streptococcal infection usually has subsided before the onset of these late complications However antibody studies have proved to be of value in establishing the occurrence of a recent streptococcal infection Thus, the titers of antistreptolysin O antistreptokinase and antihyaluronidase are still increasing at the time of onset of most cases of rheumatic fever and in doubtful cases this immunologic information may be of importance in supporting the clinical diagnosis

TREATMENT

Prior to the introduction of the sulfonamide drugs, the methods available for treatment of streptococcal infections were generally unsatisfactory Hemolytic streptococci *proved to be among the most susceptible of* all pathogenic bacteria to the bacteriostatic action of sulfanilamide and the discovery of this drug began a new era in the management of streptococcal disease The effect of the new drugs was most dramatic in the highly fatal forms of infection e g meningitis and septicemia which were seldom treated successfully by older methods The number of effective therapeutic agents has been increased by the subsequent discovery of penicillin and some of the other antibiotic drugs such as the tetracyclines since here again streptococci are among the most susceptible of all bacteria

Penicillin in constrast with the sulfonamides is bactericidal for streptococci and the use of this agent is the treatment of choice in most cases of severe streptococcal disease When penicillin is used in adequate dosage for a sufficient period of time it is possible to eliminate the organisms from the upper respiratory tract so that they are no longer recoverable on culture Sulfonamides, on the other hand, act by retarding the multiplication of streptococci which allows the natural host defenses to control the infection However, viable streptococci usually remain in the respiratory tract and the sulfonamides are not effective in eliminating the carrier state The difference in mode of action of the two drugs is reflected in their effect on antibody formation The antistreptolysin O response of patients treated with sulfonamides is not significantly different from that of the untreated patient while the response of penicillin treated patients is markedly depressed Thus destruction of the organisms appears to reduce the antigenic stimulus

Hemolytic streptococci do not appear to be able to give rise to antibiotic resistant mutants with the ease displayed by staphylococci and certain other organisms Despite the widespread use of penicillin in the general population there has been no significant change in the penicillin sensitivity of organisms isolated from streptococcal infections Even in the laboratory it is difficult to isolate variants of group A streptococci with significant resistance to penicillin Sulfonamide resistant mutants of a few serologic types of group A streptococci became prevalent following a mass prophylaxis experiment with sulfon*amides in military populations during World* War II but subsequently the incidence of these strains has greatly decreased In general it would appear that drug resistance is not a major problem in the treatment of streptococcal disease A more important complication arises from hypersensitivity of the host to the therapeutic agent Reactions of various kinds have been encountered with the sulfonamides, and at the present time there is an increasing incidence of hypersensitivity to penicillin Since penicillin sensitivity may be expressed by an acute anaphylactoid reaction the results of administration of the drug to a sensitive individual can be serious Consequently care must be exercised in the use of penicillin particularly when it is given by parenteral injection

It has been established that the prompt use of penicillin therapy in streptococcal sore throat will greatly reduce the occurrence of rheumatic fever Prevention of rheumatic fever depends on elimination of the organisms and the sulfonamides are totally ineffec

tive for this purpose When the symptoms of rheumatic fever have already developed penicillin therapy is indicated for the destruction of any streptococci remaining in the body but it appears to have little effect on the course of the acute disease However the exquisite sensitivity of group A streptococci to antibiotics has been exploited successfully in preventing recurrence of rheumatic fever in known rheumatic subjects For this purpose either penicillin or sulfonamide is effective and the continuous use of one of these agents in doses below that required for treatment of active disease decreases the risk of acquisition of a streptococcal infection and consequently the risk of recurrence of rheumatic fever

EPIDEMIOLOGY

The incidence of streptococcal infections varies in different geographic areas They are most common in the colder climates and in the north temperate zone reach a peak incidence during the winter and the early spring The apparent rarity of streptococcal infections in tropical and subtropical regions may be due in part to the fact that the disease occurs in an atypical or subclinical form since culture surveys and antibody studies indicate that the incidence of infection is higher than had been supposed on clinical grounds Crowding plays a role in the dissemination of infection The influence of this factor is illustrated by the frequency of the disease in crowded tenements and military barracks

Transmission of streptococcal infections depends primarily on intimate contact between individuals with dissemination of the organisms from the upper respiratory tract Prior to the institution of modern methods for handling and pasteurizing milk milk borne epidemics often resulted from contamination of milk by infected employees Isolated outbreaks due to infected food are still encountered occasionally but the major problem in the spread of streptococcal disease is concerned with the transfer of organisms from the respiratory tract of one individual to another

Viable streptococci are recovered readily from a variety of sources in the immediate environment of individuals who harbor the organism Thus they are found in the dust of the room in blankets on books and other objects handled by the patient These environmental reservoirs were formerly considered to be of great importance in spread of the disease but recent detailed epidemiologic studies in a military installation throw serious doubt on their significance These studies and other aspects of the epidemiology of streptococcal infections are discussed in detail by Rammelkamp (1957) The major risk of acquiring a streptococcal infection at least under the conditions of barracks life seems to be in the proximity to a carrier who is disseminating viable organisms

After spontaneous recovery from streptococcal upper respiratory infection some individuals continue to harbor the infecting organism for long periods of time In these cases streptococci can be recovered repeatedly on culture of the nasopharynx or the throat Even when cultures are negative residual organisms may remain in lymphoid tissue as indicated by the isolation of streptococci from the deep tissues of excised tonsils The potential danger of a carrier depends on a number of factors such as the number of organisms present the biologic state of the organisms and the ease with which they are disseminated into the environment In general the ability to spread infection appears to diminish with the time which the streptococci have resided in the upper respiratory tract This decrease in infectivity is referable to qualitative changes in the organisms as well as to reduction in numbers and it has been found that in many cases streptococci tend to lose their M protein during prolonged residence in the pharyngeal tissues (Rothbard and Watson 1948)

The carrier state is not necessarily dependent on previous clinical infection During epidemic periods of streptococcal disease culture surveys of school and military populations have shown that a large proportion of the population up to one third or more may carry one of the types of group A streptococci involved in the epidemic During the pre epidemic period there is often a progressive rise in the carrier rate which precedes the occurrence of significant numbers of frank streptococcal infections Epidemics can be avoided, or terminated abruptly once they

occur, by the use of mass penicillin pro phylaxis

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16

The Pneumococci

The pneumococcus is a gram positive coccus frequently of lancet shape usually arranged in pairs or short chains The viru lent forms possess an easily demonstrable capsule Pneumococcus is nonmotile and does not form spores It is lysed by bile salts and other surface active agents It is classified into types on the basis of immunologic and chemical differences in the highly polymer ized polysaccharides which compose its cap sule It is an inhabitant of the upper respira tory tract of man and certain animals and causes infection primarily in the respiratory tract and adjacent structures especially pneu monia sinusitis otitis conjunctivitis and meningitis

Synonyms are *Diplococcus pneumoniae* and *Streptococcus pneumoniae*

HISTORY

Pneumococcus was first isolated and cul tured in 1881 by Pasteur in France and in dependently in the same year by Sternberg in New York In both instances saliva of per sons who were not suffering from a respira tory disease was injected into rabbits from whose blood the organisms were subsequently isolated Neither Pasteur nor Sternberg recog nized the relationship of the organism to disease This was demonstrated by the inde pendent studies of Frankel and of Weichsel baum between 1884 and 1886 who showed pneumococcus to be the most frequent cause of lobar pneumonia in man

Classification of pneumococcus into types began in Germany with the work of Neufeld and Handel (1909 and 1910) who observed that strains of pneumococci differ in their immunologic properties Shortly afterward Dochez and Gillespie (1913) in New York subdivided pneumococci into 3 distinct types and a 4th heterogeneous group on the basis of agglutination reactions and protection tests in mice Simultaneously Lister in South Africa reported similar findings Two of the types described by Dochez and Gillespie namely Types I and II have been associated with pneumococcal pneumonia in adults more commonly than any of the other types to gether they were responsible for about one half of all cases during the 20 year period between 1920 and 1940 During the past two decades infection caused by Type II has been very uncommon on the eastern seaboard of the United States in contrast with the earlier experience (Austrian 1959) Infor mation on type incidence in other sections of the country is not available

Type III the 3rd type described by Dochez and Gillespie is carried in the normal human pharynx more commonly than any other sin gle type of pneumococcus and is likewise a frequent cause of pneumonia and other le sions Subsequent study of the heterogeneous group IV of Dochez and Gillespie has re sulted in the recognition of more than 80 distinct pneumococcal types

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The basis for the immunologic classification of pneumococci into types was demon

strated by Dochez and Avery (1917) to reside in the elaboration during growth of so-called specific soluble substances (SSS) which constitute the capsules of the microorganisms Heidelberger and Avery (1923) showed that the specific soluble substances are carbohydrate in nature and to them and Goebel is due the greatest part of our knowledge of the immunologic and chemical properties of these capsular constituents The production of the capsular polysaccharides is essential to the pathogenicity of pneumococcus, and antibodies which protect man or animals against infection are directed against this capsular material

Many of the biologic properties of pneumococcus are similar to those of various species of streptococci especially streptococci of the viridans group It is for this reason that certain taxonomists have included pneumococcus as a species of streptococcus under the name *Streptococcus pneumoniae* though agreement on this designation is not universal The most important properties which distinguish pneumococcus from the green producing streptococci are its predilection in causing pneumonia, its virulence for laboratory animals on primary isolation and its dissolution by bile salts and other surface active agents

MORPHOLOGY

In the sputum the pus and the lungs of patients with pneumonia pneumococcus appears singly in pairs or in short chains of ovoid or lanceolate cocci When in pairs, the adjacent ends of the cocci are generally rounded with the distal ends pointed The appearance in artificial culture medium is similar except that short chains of cocci are seen more commonly especially in young cultures During the active phases of growth pneumococcus is gram positive but as the culture begins to age gram negative forms appear which retain their coccoid shape On continued incubation the gram positive forms gradually disappear and eventually are replaced entirely by gram negative cocci This is followed by further autolytic changes so that finally no formed elements but only gram negative debris can be seen

The process of autolysis can be greatly enhanced by surface active compounds On the addition of whole bile or bile salts such as sodium deoxycholate or taurocholate pneumococcus autolyzes with great rapidity the clearing of a turbid suspension of organisms occurring within a matter of minutes The phenomenon of bile solubility is due to activation of the autolytic enzymes of pneumococcus If the enzymes are first inactivated by heating the suspension of cocci at 65 C for 30 minutes, autolysis no longer takes place either spontaneously or when bile is added The mechanism of activation of the autolytic enzyme system by surface active substances has not been explained, though it seems not unlikely that activation results from alteration or removal from the cells of a normal inhibitor of autolysis

On the surface of solid media such as fresh peptone infusion agar plates to which blood has been added young cultures of the virulent organisms form smooth glistening unpigmented dome shaped colonies which are circular in outline and in general vary from 0 5 to 1 5 mm in diameter Colonies formed by Type III pneumococcus are larger and more mucoid than those produced by other types and commonly attain a diameter of from 2 to 3 mm on blood agar The greater size and more mucoid consistency of the Type III colonies are due to the larger amount of capsular polysaccharide synthesized by this type As the cultures on blood agar age autolytic changes appear The centers of the colonies collapse often leaving a small central papilla with a depressed area intervening between it and the raised outer margin of the colony

Surrounding the colonies on blood agar and becoming more apparent with continued incubation at 37° C there is a zone of alpha hemolysis showing the characteristic greenish brown color

In fluid media encapsulated pneumococci grow diffusely and tend to sediment only when the pH has fallen because of acid production This occurs in media containing relatively large amounts of glucose or other fermentable carbohydrates

NUTRITION

In the older literature pneumococcus is characterized as a fastidious" microorganism indicating difficulties in cultivation and

maintenance in the viable state These difficulties were probably multiple in nature and due not only to partial deficiencies in essential nutrients but also to improper oxidation reduction potential of the medium

The importance of the oxidation reduction potential has been clarified, especially through the studies of Dubos (1929) When peptone meat infusion broth is exposed to the air the medium becomes oxidized Under such circumstances large inocula of pneumococcus must be used to obtain growth The large inoculum is able through its metabolic activity to lower the redox potential sufficiently to permit growth whereas with a small inoculum this may not occur When the medium is reduced by placing it under reduced pressure by heating to drive off dissolved oxygen or better by addition of a reducing agent such as cysteine or thioglycollic acid growth can be initiated from a very small inoculum Media satisfactory for pneumococcal growth can be prepared readily from fresh meat infusion with the addition of any good brand of peptone The pH of the medium should be between 7 2 and 7 4 after sterilization which should be accomplished with a minimum of heating Media made with a fresh meat infusion base are in general more satisfactory than the dehydrated media which are commercially available

The optimum pH for growth of pneumococcus is stated to be 7 8 Excellent growth can be obtained over the range of pH 7 2 to 7 8 but from the practical viewpoint it is probably desirable to use media of pH 7 2 to 7 4 because there is less chance of deleterious alterations occurring on sterilization by heat at lower pH values

All of the nutritional factors required for growth of pneumococcus have not been determined However a partially defined medium has been prepared by Adams and Roe (1945) which supports the growth of many but not all strains This medium is basically that designed by Bernheimer *et al* (1942) for the cultivation of Group A streptococci with the addition of asparagine and choline which have been shown to be essential for growth of most pneumococcal strains tested In addition to an acid hydrolysate of casein supplemented by l cystine and l tryptophane the medium contains the following accessory growth factors biotin nicotinic acid pantothenic acid choline pyridoxine thiamine riboflavin adenine and uracil Of these the first 4 are known to be essential In common with Group A streptococci pneumococcus has been found to require large amounts of glutamine Immediately before inoculation sodium bicarbonate is added to provide CO which is essential for the initiation of growth and the redox potential is lowered by the addition of a reducing agent such as thioglycollic acid Glucose is used as a carbon source since pneumococcus derives almost all of its energy requirements from anaerobic glycolysis Table 1 which is taken from the paper by Adams and Roe lists the components of the partially defined medium and the method of preparation Further work is required to find out if all of the accessory growth factors listed are essential for pneumococcal growth and to determine the amino acid requirements

Defined media such as that described are especially useful for chemical investigations of bacteria for example the preparation of capsular polysaccharides or somatic proteins because all of the constituents of the medium are dialyzable and can be eliminated by this means

For routine cultivation of pneumococcus it is preferable to use complex media such as peptone fresh meat infusion broth to which blood has been added Growth of all strains can be obtained autolysis is less rapid than in the defined medium described and cultures can be stored in the refrigerator for prolonged periods of time The presence of blood is particularly important for storing cultures Presumably this is because the catalase present in the red cells destroys hydrogen peroxide produced by pneumococcus Pneumococcus contains neither catalase nor peroxidase and in consequence H O accumulates in its environment if air is present, in an amount probably great enough to affect the viability of the organisms In any case although blood does not improve the growth of pneumococcus when added to a good medium its presence favors preservation of viability of the organisms on storage

PHYSIOLOGY

As noted above pneumococcus derives most of its energy requirements from the fer

TABLE 1 COMPOSITION AND METHOD OF PREPARATION OF A PARTIALLY DEFINED MEDIUM FOR PNEUMOCOCCUS*

Basal Medium—for 1 liter of medium	
Acid hydrolysate of casein	200 ml of 10% solution
l Cystine	150 mg
l Tryptophane	20 mg
KCl	3 Gm
$Na_2HPO_4 \cdot 12H_2O$	7 5 Gm
$MgSO_4 \cdot 7H_2O$	0 5 Gm
Distilled water to make	900 ml

Adjust pH to 7 5 heat to boiling filter and tube in 9 ml amounts or appropriate multipl Autoclave

Solution 1—vitamin mixture for 12 5 liters	
Biotin	0 015 mg
Nicotinic acid	15 0 mg
Pyridoxine	15 0 mg
Calcium pantothenate	60 0 mg
Thiamine	15 0 mg
Riboflavin	7 0 mg
Adenine sulfate	150 0 mg
Uracil	150 0 mg

Dissolve in 100 ml of distilled water and sterilize by filtration Store in refrigerator

Solution 2—salt mixture for 50 liters	
$FeSO_4 \cdot 7H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	50 mg
$ZnSO_4 \cdot 7H_2O$	50 mg
$MnCl_2 \cdot 4H_2O$	20 mg
HCl concentrated	1 ml

Dissolve in 100 ml of distilled water and sterilize by boiling

Addition mixture per liter of medium	
Vitamin mixture (solution 1)	8 0 ml
Salt mixture (solution 2)	2 0 ml
Glucose (20% solution)	10 0 ml
Glutamine	200 mg
Asparagine	100 mg
Choline	10 mg
$CaCl_2 \cdot 2H_2O$	10 mg
Distilled water to make	50 ml

Sterilize by filtration and store in refrigerator Add 0 5 ml to each 9 ml of basal medium This addition mixture should not be kept longer than a few weeks since the glutamine is unstable Solutions 1 and 2 appear to keep indefinitely

Bicarbonate—thioglycollate mixture	
Thioglycollic acid	10%

Add 1 ml of thioglycollic acid to 9 ml of sterile distilled water mix well and heat in boiling water bath for 10 minutes

Bicarbonate Weigh 200 mg samples of sodium bicarbonate into test tubes and autoclave

Add 10 ml of sterile distilled water to a test tube containing bicarbonate and dissolve the latter Then add 0 2 ml of 10 per cent thioglycollic acid and mix well immediately Add 0 5 ml of the mixture to each 9 5 ml of medium This bicarbonate thioglycollate mixture is unstable and must be made up and added to the medium just prior to inoculation

* From Adams M H and Roe A S 1945 A partially defined medium for cultivation of pneumococcus J Bact 49 401

mentation of glucose Practically all of the glucose metabolized can be accounted for by the lactic acid which accumulates in the medium during growth The metabolism of pneumococcus is thus essentially anaerobic However it can grow in the presence or the absence of oxygen and hence has been classified as aerobic or facultatively anaerobic Growth is somewhat better under aerobic conditions even though pneumococcus does not possess a complete cytochrome system or catalase Better growth under aerobic conditions apparently is due to the capacity to take up oxygen through a flavin containing enzyme system Under these conditions H_2O_2 is produced by the auto-oxidation of reduced flavoprotein As previously noted H_2O_2 accumulates in the medium because pneumococcus possesses neither catalase nor peroxidase

One of the most important factors limiting the growth of pneumococcus is the lowering of the pH of the medium due mainly to the accumulation of lactic acid although with some strains significant amounts of formic and acetic acid may be produced (Friedemann 1938) Massive growth can be obtained by supplying a large amount of glucose and neutralizing the lactic acid with NaOH as it is formed For the preparation of the capsular polysaccharides neutralization with NaOH should be avoided Even though alkali is added cautiously and with very careful stirring of the culture during addition it has been shown that degradation of the polysaccharides is likely to occur (Heidelberger *et al* 1950)

IDENTIFICATION

Members of the heterogeneous group of gram positive cocci collectively described under the term viridans streptococci are the only bacteria which are commonly confused with pneumococcus However a single test may be used to separate them namely bile solubility Pneumococcus is bile soluble whereas the viridans streptococcus is not This test is very reliable provided that the conditions are proper and appropriate controls are used In general it is advisable to remove the living organisms by centrifugation from the medium in which they are grown especially if it contains blood or other protein materials The organisms should be suspended in isotonic saline adjusted so that the pH is in the neutral range Under these circumstances pneumococci go into solution with great rapidity either at room temperature or at 37° C on the addition of an equal volume of 10 per cent bile Dissolution is faster at 37° C and if the suspension has not cleared within 30 minutes at this temperature it is most unlikely that the microorganism in question is pneumococcus Individual bile salts such as sodium deoxycholate or sodium taurocholate in a concentration of 1 per cent may be substituted for whole bile in carrying out the solubility test

In recent years use has been made of bile containing disks to distinguish pneumococcus from streptococci A bile disk is placed on a blood agar plate on which the culture has been streaked If pneumococcus is present its growth is inhibited in a zone surrounding the disk whereas the growth of streptococci is unaffected

Disks containing optochin (ethylhydrocupreine hydrochloride) can be used in a similar way Growth of pneumococcus is inhibited by optochin but streptococcal growth is not affected unless very high concentrations of the drug are used

The fermentation of inulin has been used as a differential test to distinguish pneumococci from streptococci While it is true that inulin fermentation is a property of pneumococcus it is not a reliable test when used by itself since certain streptococci especially those of the *salivarius* group share this capacity

Another property which is of great use in identifying pneumococcus is its virulence for mice on inoculation Streptococci of the viridans group are avirulent for mice whereas most strains of pneumococci are highly virulent even on primary isolation However there are important exceptions since certain commonly encountered pneumococcal types for example Type XIV are of low mouse virulence

VARIATION

On cultivating encapsulated pneumococci in nutrient broth to which antipolysaccharide

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the culture medium under appropriate environmental conditions is taken up by the growing cells incorporated into their genetic structure replicated and transmitted to the descendants Therefore transformations in pneumococci have great biologic significance because it was by these reactions that it was demonstrated originally that DNA possesses genetic specificity and a method was provided for studying the characteristics of the genetically active material

DNA extracted from pneumococci or other cells is not a single molecular species but rather a mixture of many deoxyribonucleic acids which differ from one another both in their content of purine and pyrimidine bases and their sequence in the nucleotide chains Depolymerization effected by enzymatic chemical or physical means destroys the specificity of DNA as evidenced by the loss of its ability to cause genetic transformation

The mechanisms which enable bacterial cells to take up free DNA from the environment and incorporate it into their genetic constitution is unknown The long threadlike molecules with molecular weights estimated by different investigators to be from 1 000 000 to 16 000 000 are transported across the cell wall and the cytoplasmic membrane and having gained access to the interior of the cell are incorporated into the nuclear apparatus by a process which appears to be analogous to crossing over in genetic exchanges in higher species

The usefulness of transformation reactions in genetic studies has been greatly extended by the discovery of additional hereditary characteristics of pneumococci and other bacterial species which can be altered by them The demonstration by Hotchkiss (1951) that drug resistance can be transferred to sensitive cells by DNA from resistant mutants has provided a series of genetic markers that permit quantitative studies to be carried out with relative ease (Hotchkiss 1957) Other pneumococcal transformations include alterations in the quantity of SSS produced changes in the serologic type of M proteins capacity to produce an adaptive enzyme mannitol phosphate dehydrogenase changes in R colonial morphology It has also been demonstrated (Bracco *et al* 1957) using streptomycin resistance and resistance to optochin (ethylhydrocupreine) as genetic markers that transformations mediated by the respective DNA s can take place reciprocally between pneumococci and distantly related streptococci The efficiency of interspecies transformation is less than the intraspecies reaction presumably because of less accurate fit of heterologous DNA in the receptor choromosome (Krauss and MacLeod 1963)

Until recently genetic transformations have been observed only under artificial conditions in the mouse or in the test tube However the experiments of Ottolenghi and Hotchkiss (1962) show that pneumococcal cultures release genetically active DNA during growth and that recombinants can be recovered from mixed growing cultures in the test tube In addition it has been demonstrated (Ottolenghi and MacLeod 1963) that genetic exchange can occur between two genetically distinguishable populations of pneumococcus growing together in the same living host the mouse These studies support strongly the idea that genetic exchange by way of transformation is a natural mechanism of recombination

ANTIGENIC STRUCTURE

Pneumococcus can be divided into 75 or more types on the basis of differences in the capsules which surround the cells The capsules are composed of highly polymerized polysaccharides which are immunologically distinct for each type Extensive studies carried out especially by Heidelberger Goebel Avery and by Stacey and his associates have provided a chemical basis for the immunologic specificity of the capsular polysaccharides of a number of types Table 2 shows certain of the chemical properties of polysaccharides of Types I II III VIII XIV and XVIII

It has been repeatedly noted that the capsular polysaccharides of pneumococci are antigenic in certain species such as man and mouse whereas in other species such as rabbit they are not antigenic but behave rather as haptenes However antipolysaccharide antibodies in very high titer can be prepared readily in rabbits by injecting them with

antibodies have been added after a few serial transfers many of the cocci will be found to be devoid of capsules (Stryker 1916) For example when pneumococcus Type I is grown in the presence of Type I antipneumococcal serum encapsulated Type I pneumococci disappear and are replaced by non encapsulated forms With loss of the capsule the organisms lose type specificity and virtually all of their pathogenicity

On the surface of an agar medium encapsulated pneumococci form characteristic smooth glistening colonies The pneumococci composing the smooth colonies are referred to as *mucoid smooth* or *S* organisms With disappearance of the encapsulated forms such as occurs on cultivation in homologous antiserum the colonies formed on agar lose their glistening appearance and tend to have a finely granular or roughened surface The organisms composing these colonies are spoken of as *rough* or *R*

A third form characterized by colonies with a markedly roughened surface has been described by Dawson (1934) to appear following prolonged incubation of cultures planted on the surface of agar plates Whether grown in fluid or on solid media the organisms of such strains are arranged in chains that may contain several hundred cocci In strains of *viridans* streptococci where colonies having a very rough surface are commonly seen, the cocci also occur in chains of great length The markedly roughened surface seems to be caused in both cases by the coiling and the piling up of the long coccal chains

It has been customary in the past to speak of anti S serum as "causing reversion from S to R thereby inferring that anti S serum in some way depresses capsular synthesis and that anti R serum stimulates it This is an incorrect view Any pneumococcal culture although superficially homogeneous contains a large number of variants and S cultures throw off R mutants at a fairly constant rate It has been shown experimentally that if a mixture of S and R cells is inoculated into broth containing anti S serum R cells have a selective advantage over S and conversely when such a mixture is grown in anti R serum S organisms have a selective advantage over R forms

When certain cultures of R pneumococci are grown in broth containing anti R serum or when the R culture is inoculated into mice the R pneumococci disappear and are replaced by S pneumococci of the same serologic type as that from which the R strain was derived by mutation and selection With some R cultures this does not occur R cultures which revert" to the S form either contain S organisms in numbers so small that they are undetectable unless they are cultivated in a selective environment or else back mutation from R to S has occurred

The enhancement of virulence which occurs upon repeated animal passage is due to a similar process of selection The less virulent forms are destroyed by the host the more virulent are able to survive and thus are specifically selected Selection of drug resistant variants or mutants by cultivating the organisms in increasing concentrations of a particular drug may also be cited as another example of the same process

Variation in pneumococcus can also be studied by means of *transformation reactions* As shown originally by Griffith (1928) pneumococcus of one specific type can be transformed to another specific type by way of the R variant In his original experiments Griffith obtained R cells from a culture of pneumococcus Type II by cultivation in Type II antiserum Then the living R cells derived from Type II were subcutaneously inoculated into mice along with large numbers of heat killed Type III S cells On death of the mice cultures of the heart blood showed the presence of living Type III S cells In other words R cells derived from a strain that originally synthesized Type II capsular polysaccharide had been transformed so that they produced the Type III capsule This alteration in polysaccharide synthesis resulted from a permanent genetic change in the recipient cells

Subsequently it was shown by Avery MacLeod and McCarty (1944) that type transformation can be effected by purified preparations of highly polymerized deoxyribonucleic acids (DNA) DNA from S cells of a particular type when applied to R cells derived from another type cause the latter to synthesize capsular polysaccharide of the type from which the DNA was prepared. Exogenous DNA which has been added to

whole encapsulated pneumococcal cells Recent studies show that the isolated polysaccharides behave not as haptenes in rabbits but as weak primary antigens if injected in appropriate small doses (2 to 20 mcg) Increase in dosage obliterates the immune response in rabbits as it does in the case of the mouse in which species also the effective antigenic dose is critically small (0 2 to 5 0 mcg) Larger doses of polysaccharide result in a state called immunologic paralysis by Felton and Ottinger who first described the phenomenon in the mouse (1942) Paralysis or immunologic unresponsiveness is specific for the polysaccharide injected and does not affect demonstrably the response to unrelated antigens Its mechanism is not yet understood

In both mouse and rabbit the isolated capsular polysaccharides are only weakly antigenic as shown by the very low antibody titers resulting from primary immunization as well as the inability to yield an anamnestic response when injected a second time The ease with which immunologic unresponsiveness can be induced in mouse and rabbit by moderate increases in the dose of polysaccharides probably is related to their low order of antigenicity in these two animal species In rabbits that have developed antipolysaccharide antibodies in high titer following hyperimmunization with intact living or killed pneumococcal cells immunologic paralysis does not occur when large doses of homologous polysaccharide are injected On the contrary such previously immunized rabbits develop a brisk secondary immune response

The somatic portion of the cells although antigenically similar in all types is not identical Antisera prepared against R pneumococci derived from one type agglutinate homologous R organisms to higher titer than heterologous R cells This is due in part to the presence of prolaminelike M proteins which are antigenically dissimilar in pneumococci of different capsular types and in the R organisms derived therefrom (Austrian and MacLeod 1949) In nature cells of a particular capsular type commonly possess an M protein characteristic for that type However capsular polysaccharide and M protein can vary independently of one another as shown particularly by transformation reactions Pneumococcal M proteins have chemical properties similar to M proteins of Group A streptococci but are immunologically distinct

As in β hemolytic streptococci the somatic portion of pneumococcus contains a C or cellular carbohydrate described by Tillett Goebel and Avery (1930) which is immunologically as characteristic of pneumococcus as a species as are the C carbohydrates of the Lancefield groups of streptococci However the C carbohydrate of pneumococcus has not been used as a means of classifying pneumococci

Goebel and Adams (1943) showed that the C carbohydrate forms a portion of the Forsmann (heterophile) antigen of pneumococcus Recent studies of Liu and Gotschlich (1963) have resulted in the identification of the components making up about 80 per cent of the weight of C polysaccharide There are 4 major amino acids (lysine serine glutamic acid and alanine) and 4 amino sugars (D glucosamine D galactosamine 6 phosphate muramic acid and muramic acid phosphate) Almost 35 per cent of the total weight of the polysaccharide is accounted for by D galactosamine 6 phosphate This sugar has not been found previously in bacterial cell walls

Tillett (1928) demonstrated that rabbits immunized with heat killed R pneumococci develop resistance to infection with virulent encapsulated pneumococci of Types I II and III Subsequently Dubos (1938) extracted a soluble antigen from pneumococcal cells which caused the production in rabbits of antibodies capable of protecting mice against infection with both homologous and heterologous types The nature of the antigen responsible for the broad immunity studied by Tillett and by Dubos is unknown although there is evidence that the C polysaccharide may be involved (Enders Wu and Shaffer 1936) Antibodies to M protein are not protective However it is worth emphasizing that non type specific resistance to infection obtained by the methods described above is slight when compared with the high degree of immunity afforded by type specific antibodies and probably plays a minor part in protection against pneumococcal infection

TABLE 2 PROPERTIES OF THE SPECIFIC CAPSULAR POLYSACCHARIDES OF SIX PNEUMOCOCCAL TYPES

TYPE	$[\alpha]_D$	ACID EQUIVALENT	MOLECULAR WEIGHT	N	REDUCING POWER ON HYDROLYSIS AS GLUCOSE	CONSTITUENTS IDENTIFIED	COMMENT
				per cent	per cent		
I[1]	+265 to +277	650	171 000	4 6	30	Galacturonic acid (30%) N acetyl glucosamine acetic acid	
II[3]	+60	1030	504 000[2]	0 2 (none 6b)	95	D glucose D glucuronic acid L-rhamnose 1 3 7	Cross reacts with K friedlander Type B All glucose in form 1 4 6 branch points (3a) Cross reacts with lung galactan (3c) and gum arabic (3d)
III[4]	−33 to −37	3 0	141 000[2]	0 05	85	Glucuronic acid glucose 1 1	Type III antiserum cross reacts with Type VIII polysaccharide and vice versa. Oxidized cotton cross reacts with Type III and VIII horse sera (4b) Cellobiuronic acid is the structural unit of Type III
VIII	+1 1	703	140 000	none	87	D glucose D galactose D glucuronic acid 2 1 1	Cellobiuronic acid in common with Type III
XIV[6]	+12 5	—*		2 0	84	D galactose N acetyl D glucosamine D glucose	Type XIV antiserum cross reacts with blood group substances especially A and with lung galac s (3c)
XVIII[7]	+86 to +89	—**		0 3	58	D glucose L-rhamnose phosphate 5 1 1	

Contains no acid groups
** Contains phosphate bound in secondary linkage

[1] a. Heidelberger M Kendall F E and Scherp H W J Exp Med 1936 64 559
b Westphal O Immunochemie in Physiologische Chemie Ed Flaschentrager and Lehnartz Springer 1957
Molecular weights as given by Stacey M Endeavour 1953 1 38
[3] a Butler K and Stacey M J Chem Soc 1955 Part II 1537
b Record B R and Stacey M J Chem Soc 1948 Part II 1561
c Heidelberger M Dische Z Neely W B and Wolfrom M J Am Chem Soc 1955 77 3511
d Heidelberger M Avery O T and Goebel W F J Exper Med 1929 49 847
[4] a Reeves R E and Goebel W F J Biol Chem 1941 139 511
b Heidelberger M and Hobby G L Proc Nat Acad Sci 1942 28 516
c Heidelberger M MacLeod C M Markowitz H and DiLapi M M J Exper Med 1951 94 359
[5] a Goebel W F J Biol Chem 1935 110 391
b Heidelberger M Kabat E A and Mayer M M J Exper Med 1942 75 35
c Jones J K N and Perry M B J Am Chem Soc 1957 79 2787
[6] a. Goebel W F Beeson P B and Hoagland C L J Biol Chem 1939 129 455
b Heidelberger M Lectures in Immunochemistry Academic Press New York 1956
[7] Markowitz H and Heidelberger M J Am Chem Soc 1954 76 1317

tions to this generalization because certain types of which XIV is a good example have a low order of virulence for mice and it is not enhanced on mouse passage Despite its low mouse virulence Type XIV is one of the common causes of pneumococcal pneumonia in children

Similar observations have been made in the rabbit with pneumococcus Type III which although highly virulent for man and mouse is almost avirulent for the rabbit However an occasional strain of Type III has been described which possesses pathogenic properties for rabbits A satisfactory explanation for the difference in virulence of these strains of pneumococcus Type III has not been found

Both dogs and monkeys have been used extensively for the study of pneumococcal lobar pneumonia produced by experimental inoculation by the intratracheal route and under appropriate conditions both are highly susceptible Felines are relatively insusceptible to experimental infection and birds are especially resistant

It is apparent from the observations on pathogenicity described above that the data obtained from studies of one animal species cannot be transferred to another

FACTORS INVOLVED IN PATHOGENICITY OF PNEUMOCOCCUS

Pneumococcus is a good illustration of a bacterial species that produces disease apparently solely through invasive properties in other words because of the capacity to invade and multiply in living tissues without evidence that soluble toxins in the usual sense play a part *Cl botulinum* at the other end of the scale exerts its pathogenicity entirely through a potent exotoxin which is produced outside the body and causes disease on absorption through the gut *Cl botulinum* has no invasive capacity whatsoever Between these two extremes of purely invasive and purely toxigenic pathogens are many species that possess both capacities in varying degrees

The search for a toxin produced by pneumococcus which can account for its disease producing capacity has been unsuccessful It is true that two toxic principles have been identified but since neither of them is liberated in detectable amount except on autolysis of the bacterial cells it is doubtful that either plays a significant part in pneumococcal pathogenicity

Pneumolysin an oxygen labile or O hemolysin is liberated from pneumococcus especially on autolysis (Cole 1914) It is related serologically to the O hemolysins produced by hemolytic streptococci *Cl tetani* and *Cl welchii* as shown by Todd (1934) However it should be noted that hemolysis is never a feature of even overwhelming pneumococcal infection and also that in the case of the 3 toxigenic species noted above which produce similar O hemolysins it has not been proved that these substances play a significant part in disease caused by the respective species

Under conditions of autolysis there is also liberated from pneumococcus a purpura producing principle whose effect can be demonstrated both in the skin and the internal organs of experimental animals injected with sterile pneumococcal autolysates Once again however purpura or other hemorrhagic incidents are not seen except in the rarest instances in pneumococcal infection whether in man or other animals so that the purpura producing principle would appear to have little significance in the pathogenicity of pneumococci

So far as we know at present, pneumococci produce disease and death solely through their capacity to multiply in the tissues However it is not improbable that a toxin is produced in the tissues which differs in nature from any previously studied and that its demonstration requires new methods

Another possible mechanism whereby such a rapidly growing pathogen might produce tissue damage is through the production of local or general acute deficiencies in one or more essential metabolites whether vitamins amino acids purines or constituents of other cell components The demands of bacterial cells having a generation time of from 20 to 30 minutes on available supplies of essential metabolites in the animal body must be great indeed and conceivably the microorganisms might compete for limited supplies of such materials on a more favorable basis than the

TABLE 3 DISTRIBUTION OF PNEUMOCOCCAL TYPES IN ADULTS WITH LOBAR PNEUMONIA

PNEUMOCOCCAL TYPE	NUMBER OF CASES	PER CENT OF TOTAL
I	1 063	28 6
II	425	11 4
III	*500*	*13 5*
IV	131	3 5
V	298	8 0
VI	66	1 8
VII	240	6 5
VIII	287	7 7
Total I VIII	*3 010*	*81 0*
All other types	703	19 0
	3 713	100 0

DISTRIBUTION

Pneumococcus has a world wide distribution It is a normal inhabitant of the nasopharynx of man under all climatic conditions In many parts of the world Types I and II have been reported as the most frequent causes of human disease although in normal people other types are carried more commonly in the pharynx Among other animal species, guinea pigs, monkeys and rats are the only ones that are known to harbor pneumococci commonly These species have little or no importance as reservoirs of pneumococci so far as human disease is concerned However epizootics of pneumococcal pneumonia occur among monkeys in captivity and in the guinea pig pneumococcus Type XIX causes one of the most frequent and fatal epizootic diseases of this species None of the other types is of importance in causing disease in guinea pigs though they may become carriers if other types are experimentally implanted in the nosapharynx In rats severe epizootics have been caused by pneumococcus Type II (Mirick *et al* 1950)

PATHOGENICITY AND HOST RANGE

Infections in man can be caused by any of the more than 75 serologic types of *pneumococcus* However, of this large number of types a few account for most human disease Types I II and III cause approximately one half of all the cases of lobar pneumonia in adults in different parts of the world (Heffron, 1939), with 8 types causing about 75 to 80 per cent of the total number of infections Table 3 which is taken from the summaries of Heffron shows the incidence of lobar pneumonia caused by various types in 3 large cities in the north eastern United States

Following the advent of chemotherapy for pneumococcal infections in the latter half of the 1930s typing of pneumococci from patients with pneumonia was abandoned in most parts of the world because the effectiveness of the new drugs did not appear to be related to the immunologic type causing disease Therefore it is not known whether the type distribution is the same now as it was before antimicrobial drugs were introduced In this respect the data collected by Austrian (1959) on pneumococcal infections in New York City between 1952 and 1957 are of considerable interest In Austrian s series of 1,322 adult patients, pnenmococcus Type II was found only 7 times an incidence of about 0 5 per cent whereas in series of cases collected before 1935, Type II accounted for as much as 11 per cent of adult infections

It should be noted that the figures given in Table 3 are for cases of pneumonia having anatomically a lobar distribution Pneumococci are also the most frequent causative agents of bronchopneumonia

In children below the age of 12 the distribution of pneumococcal types in lobar pneumonia differs from that in adults with Types XIV I VI V VII and XIX *in that order* causing more than half the cases (Heffron) It is of interest that the capsular polysaccharide of Type XIV is immunologically related to blood group A substance (Beeson and Goebel 1939)

Among laboratory animals the mouse is the most susceptible species although rats and rabbits are also highly susceptible to experimental inoculation The virulence of most types for mice can be enhanced by repeated passage due to the selection by this means of the most virulent mutants from a mixed population of cells With many types as few as 1 to 5 cocci inoculated intraperitoneally into mice will cause death within 48 hours However there are important excep

tions to this generalization because certain types of which XIV is a good example have a low order of virulence for mice and it is not enhanced on mouse passage Despite its low mouse virulence Type XIV is one of the common causes of pneumococcal pneumonia in children

Similar observations have been made in the rabbit with pneumococcus Type III which although highly virulent for man and mouse is almost avirulent for the rabbit However an occasional strain of Type III has been described which possesses pathogenic properties for rabbits A satisfactory explanation for the difference in virulence of these strains of pneumococcus Type III has not been found

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cells of the host with a resulting acute deficiency for the host cells Other means by which a primarily invasive organism such as pneumococcus causes tissue damage might be suggested but would serve only to emphasize our state of ignorance

Whatever may be the ultimate means by which pneumococcus produces damage to tissues a fact of great significance is that most of the pathogenic power of this species is exerted through possession of a nontoxic surface component—the pneumococcal capsule All virulent strains of pneumococcus possess a capsule which forms a protective mantle about the cells The sole function of the pneumococcal capsule appears to be to *delay or prevent ingestion of pneumococci by* the phagocytic cells of the body The free isolated polysaccharides which compose the capsules in the different types are nontoxic on injection in even very large doses Evidence of the role of the capsule in pathogenicity has been obtained in a variety of ways a number of which will be described

Upon immunization of animals by injecting them with killed encapsulated pneumococci, antibodies are formed which are able to protect other animals infected experimentally with the same type For example a serum prepared by immunizing rabbits with pneumococcus Type I protects against experimental infection with living Type I pneumococci but will not protect against infection with Type II pneumococcus In other words the protective antibodies are type specific

The capsular polysaccharides of several types of pneumococcus have been prepared in relatively pure form as previously noted It has been shown that the protective capacity of a serum depends on the amount of antibody present which is capable of reacting with the capsular polysaccharides By the addition of an appropriate amount of purified specific capsular polysaccharide to a highly protective serum all of the anticapsular antibodies can be precipitated and removed A serum absorbed in this way loses its protective power *Anticapsular* antibodies can also be absorbed from a serum by permitting it to react with whole encapsulated pneumococcal cells and here again the protective power is removed at the same time However one cannot conclude from the results of absorption with whole bacterial cells that it is the anticapsular antibodies that are of importance in protecting against infection since other antibodies reacting with other components of the pneumococcal cell may be removed simultaneously On the other hand absorption of serum with purified capsular polysaccharides shows unequivocally that it is the *antibody to this material that is of importance* in protection against infection and at the same time demonstrates the importance of the capsule in the pathogenicity of the organism

Loss of the capsule from R cells which has resulted because of mutation deprives the cells of most of their virulence although *not all With fully virulent encapsulated* strains a dilution of broth culture containing from 1 to 5 viable organisms (1×10^{-8} ml of culture) will kill mice following intraperitoneal injection With R organisms selected from the same strain by growing it in anti S serum 0 5 to 1 0 ml of culture is necessary to cause death or approximately *100 000 000 to 200,000 000 living R organisms* Therefore loss of the capsule has decreased virulence by a factor of roughly 100 000 000 In mice in the case of Types II III and VII virulence is related to the *quantity* of SSS formed by different strains of the same type (MacLeod and Krauss 1950) For example strains of Type II that produce *small amounts of SSS II are less virulent for* mice than strains that form larger amounts

The quantity of SSS synthesized by strains of pneumococci is under genetic control This *can be shown by study of spontaneous* mutants which have different synthetic capacities for a particular polysaccharide and also by genetic transformation reactions carried *out with DNA extracted from* them In the final analysis therefore the virulence factor for a pneumococcal strain can be considered as the gene that controls the amount of capsular polysaccharide synthesized per cell The gene concerned may be expected to vary from one mutated strain to another within a given type as well as from one *type to another* because of the complex nature of the capsular polysaccharides and because of differences in chemical composition among the various types Alteration in the amount or activity of one out of a number of enzymes involved in polysaccharide syn-

thesis may cause diminished polysaccharide formation and result in decreased virulence

The antiphagocytic property of the pneumococcal capsule can be demonstrated in vitro by mixing R and S pneumococci respectively with heparinized normal human blood Phagocytosis of R cells takes place rapidly whereas the leukocytes have a limited capacity to phagocytose the S forms However if a small amount of type specific antiserum is added the S cells are taken up rapidly by the leukocytes The combination of specific antibody with the capsular polysaccharide removes its antiphagocytic property The antiphagocytic action of the capsule appears to be mechanical and not to be caused by a toxic effect on the leukocytes The detailed studies of Wood and his co workers (see review by Wood 1951 52) indicate that when phagocytosis experiments are carried out on smooth surfaces such as glass or wax, encapsulated pneumococci are pushed about by the leukocytes which have difficulty in embracing the organisms within the pseudopodia because the pneumococci slide away over the smooth surface On the other hand on a roughened surface such as blotting paper filter paper fibrin films or fixed sections of lung the leukocytes take up encapsulated pneumococci fairly readily even in the absence of specific antibody because the bacteria can be pinned against obstructions on the roughened surface and then enclosed in the pseudopodic extensions Intracellular digestion ensues Wood has shown that the nature of the surface influences phagocytosis in vivo also

If antibody is added to the mixture of encapsulated pneumococci and phagocytes the nature of the surface on which the experiment is carried out makes little difference The combination of antibody with the capsule appears to make the pneumococci sticky since now they adhere to the leukocytes and are readily ingested

From the foregoing observations it should be apparent that the presence of type specific antibody is not a prerequisite for phagocytosis of encapsulated pneumococci In the absence of antibody phagocytosis is relatively slow but it is enhanced enormously when antibody is added

The studies of Dubos and Avery on an enzyme which specifically digests the capsular polysaccharide of pneumococcus Type III both when the polysaccharide is in the isolated purified state and when it is present on the surface of the living virulent cells provide further evidence for the importance of the capsule in pathogenicity of pneumococci (Dubos 1939 1940) From the soil of a cranberry bog an aerobic sporulating bacillus was isolated which when grown in a medium containing Type III polysaccharide as the sole carbon source is induced to form an enzyme that has the capacity to hydrolyze specifically Type III capsular polysaccharide but none of the other pneumococcal polysaccharides In the test tube the Type III enzyme digests and removes the capsule from either dead or living Type III pneumococci moreover the enzyme when injected as long as 18 hours after the experimental infection of mice rabbits or monkeys with pneumococcus Type III prevents the death of the animals The sole action of the S III enzyme is to remove the pneumococcal capsule by digesting it with the result that the decapsulated bacteria become highly susceptible to phagocytosis It should be emphasized that digestion of the Type III capsule from the living organisms in vitro in no way affects their viability although as long as active enzyme is present, the capsular material is digested as rapidly as it is produced However when transferred to fresh medium lacking the S-III enzyme the pneumococci again produce their capsules normally

A considerable body of evidence has been accumulated therefore which emphasizes the importance of the pneumococcal capsule in pathogenicity On the other hand the part played by somatic factors has received little attention though there is evidence of their significance One of the most clear-cut illustrations of the influence of somatic factors is in the case of two well known laboratory strains of pneumococcus Type III known as A66 and SV III Strain SV III is highly virulent for rabbits whereas A66 has very little virulence even on repeated animal passage The capsular polysaccharide is immunologically identical in both strains Moreover if the capsule is switched from A66 to SV III and vice versa, by means of transformation reactions no change in virulence occurs (Shaffer Enders and Wu 1936) In other words the difference in virulence of

these strains appears to depend on the somatic portion of the cells

Pneumococcus may be of low virulence for a species because of the nature of the capsule Type XIV, for example is avirulent for the mouse on this basis R pneumococci derived from avirulent Type XIV pneumococcus become highly virulent in the mouse when transformed to pneumococcus Type II similarly when virulent Type II pneumococci are transformed to Type XIV by way of the R variant, the newly constituted Type XIV strain is as avirulent for the mouse as naturally occurring Type XIV pneumococci (MacLeod and McCarty 1942)

PATHOGENESIS OF PNEUMOCOCCAL INFECTION

Pneumococcal pneumonia is rarely a primary infection but usually follows damage to the respiratory tract caused by some unrelated agent, whether viral or chemical In most instances pneumonia is preceded by an upper respiratory infection such as common cold or influenza Therefore pneumococcal pneumonia may be classed as a complex infection This relationship to viral infections probably explains why pneumococcal pneumonia is commonest during the cold months of the year in northern latitudes, since common colds and influenza occur with greatest frequency at these times It is unlikely that the virulence of pneumococci for man varies with the seasons

It is apparent from the relation of pneumococcal pneumonia to viral infection that the normal respiratory mucosa must possess great natural resistance to pneumococcus especially when it is recalled that between 40 and 70 per cent of all normal humans are carriers of pneumococci many types of which are potentially virulent The factors normally involved in protection of the respiratory mucous membrane have not been defined and we are also ignorant of the nature of the predisposing injury caused by viruses or chemical irritants such as gases though it seems likely that actual destruction of the superficial cell layers may be important in permitting invasion of underlying tissues Epidemiologic evidence indicates that the possession of type specific antibody not only protects man against infection under natural conditions but also renders him less likely to become a carrier of pneumococci of homologous type (MacLeod *et al*, 1945) However, it is reasonably certain that, in the main normal resistance to pneumococcal pneumonia does not depend on the possession of antibodies reactive with the many types with which one comes in contact but rather on other factors including whether one happens to be a carrier of a highly pathogenic type such as Type I or II

In the healthy adult pneumococcal pneumonia characteristically involves one or more lobes or a discrete portion of them, leaving the remaining bronchopulmonary system relatively uninvolved In infants young children and the aged the lesions more commonly follow a bronchial distribution without the localized character of lobar pneumonia.

Experimental pneumococcal pneumonia has been studied in the monkey by Blake and Cecil (1920) and in the dog by Robertson and others (see Terrell *et al* 1933) In both species pneumonia can be produced successfully by administering a narcotic such as morphine to depress respiration and the cough reflex and then introducing virulent pneumococci into the trachea or directly into a portion of a pulmonary lobe by means of a fine catheter In the dog an important factor is the production of atelectasis in the portion of the lobe infected and it seems likely that atelectasis may be important also in the etiology of lobar pneumonia in man Increased bronchial secretions and edema occurring during viral infections of the upper respiratory tract may play a significant part through plugging of bronchioles and the production of areas of atelectasis In addition, the tendency for secretions to pool in the most dependent portions may explain in part why the lower pulmonary lobes are most frequently involved

PATHOLOGIC AND CLINICAL PICTURE

The lesion in the lung consists essentially of marked edema of the alveolar walls with an outpouring into the alveoli of fibrinous exudate containing large numbers of red blood cells and polymorphonuclear leuko

cytes Therefore the affected lung becomes consolidated The overlying pleura is in volved early with a serous pleural effusion as a common incident Pneumococci in large numbers can be seen throughout the inflamed area but despite their presence both in the alveoli and the alveolar septa necrosis does not take place The absence of necrosis of the alveolar septa explains why upon re covery from the disease the lung is able to return to its original state

Sputum which is usually coughed up throughout the disease is characteristically bloody or rusty the red cells being intimately mixed with the other components Large numbers of pneumococci are usually present and can be identified quickly by immunologic methods especially the Neufeld quellung or capsular swelling reaction

The onset of acute lobar pneumonia is characteristically sudden often with the oc currence of a chill and sharp pleural pain Recovery of untreated cases frequently occurs likewise with dramatic suddenness—the so-called pneumonic crisis which gen erally occurs within 5 to 10 days from the initial chill

Pneumococci can be recovered from the blood in a variable proportion of cases the reported incidence of bacteremia depending in good part on the frequency with which cultures are made and the technic followed In the large series of cases compiled by Heffron (1939) bacteremia occurred in 26 per cent Persistent and increasing bactere mia are grave prognostic signs

Before the introduction of chemotherapy empyema was the commonest complication of lobar pneumonia occurring in approxi mately 3 per cent of patients (Heffron) Adequate antimicrobial therapy has made it a rarity

The evidence indicates very strongly that spontaneous recovery from pneumococcal pneumonia is dependent on the development of type specific antibody which can be demonstrated usually in the blood at about the time of crisis However it should be re membered that the antibody demonstrable in the circulating blood represents the excess left over after combination with type specific polysaccharide present in the lung and other organs of the body In other words antibody is being formed before recovery is apparent but appears in sufficient excess at that time so that it can be detected in the blood serum

LABORATORY DIAGNOSIS

The effective use of specific serum therapy for treatment of pneumococcal pneumonia necessitated rapid and accurate methods of etiologic diagnosis Of the many methods de scribed the Neufeld quellung or capsular swelling reaction is the simplest and best Neufeld observed in 1902 that when pneu mococci of a particular type and homologous antiserum are mixed together the capsules of the organisms become greatly swollen and in this state are clearly visible under the microscope The swelling is due to combina tion of specific antibody with the capsular polysaccharide

Sputum is emulsified by drawing it re peatedly into a syringe Then it is stained by Gram s method to determine whether organ isms having the morphology of pneumococci are present Loopfuls of emulsified sputum are mixed with a loopful of the various specific antipneumococcal sera a loopful of methylene blue is added to make the somatic portion of the cells more easily visible and the preparation is examined immediately under the oil immersion lens In a positive reaction the pneumococcal capsule has a greatly swollen appearance it becomes more refractile and its border is sharply delimited from the surrounding medium The quellung reaction can be used to identify pneumococci directly in sputum in spinal fluid and in the exudates from experimentally infected ani mals and when grown in artificial culture media Under the latter circumstances young cultures should be examined since in older cultures much of the capsular material diffuses away from the pneumococci into the surrounding medium so that capsular swell ing is less striking Although wet prepara tions of sputum or exudates are generally used it should be noted that the quellung reaction can also be carried out by applying specific antiserum to dried smears on glass slides

Diagnosis can also be made by injecting mice intraperitoneally with sputum In gen eral pneumococci are much more pathogenic

for the mouse than other organisms present in sputum or saliva and consequently come to predominate within a few hours in both the peritoneal exudate and the blood of the mouse On death of the mouse which usually occurs within 16 to 48 hours following injection of sputum, some of the peritoneal exudate is removed examined microscopically after Gram staining and then typed by the quellung reaction Pneumococci can usually be recovered in pure culture from the heart blood of the infected mouse It is good practice to make cultures of the heart blood for confirmatory studies

Pneumococci can be typed also by agglutination and precipitin reactions but the simplicity and the great accuracy of the quellung reaction make it a preferable technic

Culture of the blood of patients with pneumonia is important from the diagnostic point of view but even more so as a guide in prognosis

SPECIFIC SERUM THERAPY

Pneumococcal pneumonia is one of the few infectious diseases for which effective specific serum therapy was evolved The introduction of sulfonamide drugs in 1937 and the many antibiotics since that time have entirely displaced antiserum in therapy because they are more effective and much easier to use However a description of serum therapy is given because of its historical interest as well as the general principles which it illustrates The basis for serum therapy was laid in the fundamental studies of Avery Chickering Cole and Dochez (1917) who first showed convincingly that the antiserum to be used must be type specific that antibody must be given in adequate amounts and that it is most effective when administered early in the course of the disease Applied originally to treatment of pneumonia caused by Types I and II highly potent antisera later became available commercially for pneumonia caused by the majority of pneumococcal types (for summaries see Bullowa 1937, and Lord and Heffron 1938)

The earliest antisera were prepared by the immunization of horses with killed pneumococci and were administered intravenously in unconcentrated form Subsequently various methods were developed for refining and concentrating the antibody globulins in the crude serum which greatly facilitated treatment The introduction of rabbits for preparing antipneumococcal serum for therapeutic purposes was another advance since in general higher titers of antibody could be obtained (Horsfall *et al* 1937)

The dosage of type specific antipneumococcal serum can be controlled by determining whether the patient s blood and tissues contain an excess of antibody Specimens of blood serum obtained at intervals after treatment can be tested for free antibody by means of agglutination reactions with suspensions of homologous pneumococci Dosage of antiserum is adjusted so that an excess of antibody is constantly present in the blood However, a more useful method is the skin test with the purified specific capsular polysaccharides described by Francis (1933) 0 1 mg of homologous specific capsular polysaccharide dissolved in 0 1 ml of saline is injected intracutaneously If circulating antibody is present a wheal and erythema reaction appears at the site of polysaccharide injection within 15 or 30 minutes The wheal and erythema are due to the combination locally of polysaccharide and homologous antibody As early as possible in the course of the disease antibody is administered intravenously in an amount sufficient to result in a positive polysaccharide skin test In most instances defervescence and the onset of recovery occur within a few hours after sufficient antibody has been given At intervals of a few hours after serum therapy was first given the polysaccharide skin test is repeated in order to make sure that antibody remains present in excess

The ability to make a rapid etiologic diagnosis by means of the quellung reaction the preparation of highly concentrated preparations of specific antibody and the control of dosage by the polysaccharide skin test together made the serum treatment of pneumococcal pneumonia highly effective On the other hand the specialized nature of the technics involved the necessity for maintaining stocks of many type specific

antisera and the constant fear of anaphylactic reactions following intravenous administration of foreign protein prevented the serum treatment of pneumococcal pneumonia from achieving general use

CHEMOTHERAPY

Of bacteria causing human infections pneumococci are among the most susceptible to the action of antimicrobial drugs More over with the exception of the sulfonamides the appearance of drug resistance has been a rare occurrence during the course of therapy and up to the present time has caused little difficulty Resistance of pneumococci to sulfonamides has been encountered especially in patients treated with inadequate dosage and in cases in which a purulent complication such as empyema was present Penicillin the tetracycline compounds and chloramphenicol are more effective agents in treating pneumococcal infections and have largely displaced the sulfonamides although the latter are considered a useful adjunct in treating cases of meningitis Pneumonia due to tetracycline resistant strains of pneumococci has been reported recently in England and the United States

For respiratory tract infections penicillin remains the drug of choice because it is bactericidal and also because the capacity of pneumococcus to develop resistance to penicillin in vivo is extremely limited There is no authenticated instance recorded of therapeutic failure in a pneumococcal infection treated with penicillin as the result of resistance of the bacteria to the drug

It is instructive to compare the mechanism of action of specific serum therapy and chemotherapy in combating pneumococcal infections Spontaneous recovery from pneumococcal pneumonia is associated with the appearance of specific antibody in the blood over and above the amount required to combine with the capsules of the organisms and thus render them susceptible to phagocytosis Therefore in the natural disease there is a competition between the capacity of the pneumococci to grow and produce SSS and the ability of the infected person to form antibody to it Treatment with specific antiserum tips the balance in favor of the host since sufficient antiserum can be given in a short time to combine with all the SSS present both that which is on the surface of the microorganisms and that which is free in the blood and the tissues

The sulfonamide drugs which are essentially bacteriostatic act by restraining the growth of the organisms until sufficient antibody has been formed in the body to assure opsonization and phagocytosis of the pneumococci The participation of specific immunity appears to be necessary for a successful outcome in sulfonamide therapy

In the case of penicillin which is a bactericidal compound when used in a full therapeutic dosage the development of specific antibody seems to play a less significant part in recovery in the natural disease in man or in experimental infections of the highly susceptible mouse (MacLeod and Stone 1945) The role of a specific antibody response in recovery following treatment with the bacteriostatic tetracycline compounds has not been studied adequately

EPIDEMIOLOGY OF PNEUMOCOCCAL PNEUMONIA

As noted above pneumococcal pneumonia occurs almost always secondary to injury to the respiratory mucosa caused by an unrelated agent such as a viral infection or an irritating gas Furthermore the virulence of the various pneumococcal types for man differs greatly Types I and II have the most pronounced human virulence since between them they cause about half of all the cases of lobar pneumonia in the adult On the other hand certain other types are encountered very rarely as the cause of pneumonia and hence can be considered to be of low human virulence Therefore it should be apparent that the chances of developing pneumococcal pneumonia depend in great part on whether or not the nonimmune individual is a carrier of one of the more highly pathogenic pneumococcal types at the time he acquires a viral infection of the respiratory tract such as the common cold or influenza There is ample evidence (summarized by Heffron 1939) that when pneumococcal pneumonia is epidemic in a community it is always associated with a high

carrier incidence of the pneumococcal types causing disease Significantly, most epidemics of pneumococcal pneumonia reported in the literature have been caused by the same types that are responsible for most cases of endemic pneumonia Types I, II, IV, V and VII Under normal circumstances the incidence of carriers of the highly pathogenic types is relatively low However, if a high carrier incidence of pathogenic types prevails at a time when viral infections of the respiratory tract are epidemic epidemic pneumococcal pneumonia is liable to occur also (Hodges and MacLeod 1946)

Chance would appear to determine whether the nonimmune individual becomes a carrier of one or more types of the pneumococci (Hodges *et al* 1946) although the immune person is less capable of becoming a carrier than the nonimmune (MacLeod *et al* 1945)

Most reported epidemics of pneumococcal pneumonia have occurred in relatively closed communities such as mental hospitals, prisons and military installations The living conditions in such circumstances appear to favor the dissemination of the more pathogenic types once the latter are introduced into the community In addition the incidence of pneumococcal pneumonia is higher in workers in certain occupations such as in steel mills and in coal mines than in the general population (Heffron 1939)

There is evidence that the normal carrier is of more importance in the dissemination of the infective types than the patient ill with pneumonia (MacLeod *et al*, 1945)

PREVENTION OF PNEUMOCOCCAL PNEUMONIA

From the observations cited in relation to the epidemiology of pneumococcal pneumonia, it seems likely that control can be achieved either by preventing the nonbacterial respiratory infections which predispose or else by specific prophylaxis of pneumococcal infections themselves A certain amount of success has been achieved through both approaches

In recent years evidence has been presented that the incidence of influenza can be greatly reduced by immunization with vaccines of influenza virus types A and B, provided that the strains of virus present in the vaccine are closely related immunologically to the strains of virus causing epidemic influenza Therefore improvement in influenza vaccines and their general use might be expected to cause a reduction not only in influenza but also in pneumococcal pneumonia which occurs secondary to influenza Similarly the development of specific immunizing preparations for other viral infections of the respiratory tract should lead to a concomitant reduction in pneumococcal infections

Repeated attempts have been made to immunize against pneumococcal pneumonia (for review see Heffron 1939) In earlier trials the vaccines consisted of heat killed pneumococci which were injected subcutaneously Although proof was lacking the general opinion of those who employed whole bacterial vaccines was that a beneficial result was obtained The evidence of Lister and Ordman (1935) in South Africa was especially suggestive of a prophylactic effect In more recent years preparations of the capsular polysaccharides have been used following the demonstration by Francis and Tillett in 1930 that the isolated polysaccharides are antigenic for man Again suggestive evidence was obtained especially through the studies of Felton (Ekwurzel *et al* 1938) that immunization of man with the capsular polysaccharides prevents pneumococcal pneumonia Most of the studies on antipneumococcal immunization have been inadequate in one or more respects especially because of failure to determine whether the apparent reduction in pneumonia was confined to the types against which immunization was practiced as well as failure to design the experiment so that adequate controls were included

Most of the deficiencies inherent in previous attempts at immunization against pneumococcal pneumonia appear to have been avoided in the studies reported by MacLeod Hodges Heidelberger and Bernhard (1945) who have presented what may be considered as reasonably conclusive evidence that immunization by subcutaneous injection of purified capsular polysaccharides can prevent pneumococcal pneumonia

Immunization was carried out in an Army

camp where Types I II IV V VII and XII pneumococcal pneumonia had been epidemic for 2 years A dose of 0 06 mg of each of the capsular polysaccharides of pneumo coccus Types I II V and VII was injected subcutaneously into half the population the remainder serving as controls In the immu nized men occurrence of pneumonia caused by Types I II V and VII ceased within 2 weeks after immunization but continued in the nonimmunized controls The incidence of pneumonia caused by pneumo occal types other than those represented in the vaccine was not affected in either group

Although Types I II V and VII pneumo cocci continued to cause disease in the non immune group the incidence was not so high as was expected on the basis of the previous 2 years experience moreover the incidence of pneumonia caused by Type XII and other types was unaffected Therefore it is likely that immunization of one half the population protects not only those who are immunized but also affords a measure of protection to the nonimmune segment Partial protection of the nonimmune portion of the population may be explained by the observation that the immunized individual is less capable of car rying homologous pneumococci in the phar ynx than the nonimmune and because of the reduction in carriers dissemination of pneumococci is reduced

Large scale immunization of a civilian population by means of specific polysac charides has not been employed for the prevention of pneumococcal pneumonia

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staphylococcus from the Greek *staphule* = a bunch of grapes

The ability of staphylococci to induce abscess formation in man was established by Garré (1885) and others who inoculated or rubbed staphylococcal cultures into the skin and recovered the organism from the localized pustules which formed

On the basis of colonial pigmentation 3 species of staphylococci were recognized *Staph aureus Staph albus* and *Staph citreus* (Rosenbach 1884 Passet 1885) Most strains isolated from human lesions produced the golden yellow *aureus* pigment whereas colonies of the usually nonpathogenic staphylococci were white (*albus*) or lemon yellow (*citreus*) However since colonies of pathogenic strains were sometimes white it became apparent that colonial pigmentation alone would not suffice for classification Studies on the physiologic and the biochemical attributes of the staphylococci also failed to provide a firm basis for species differentiation The early observations of Loeb (1903) and Much (1908) that cultures of *Staph aureus* coagulate plasma were extended and coagulase production is now the most useful criterion for identification A staphylococcus that produces coagulase is now termed *Staph aureus* irrespective of colonial pigmentation (Breed *et al* 1957) However the general problem of taxonomy of staphylococci and micrococci remains quite complex (Cowan 1962)

Staphylococcal disease although of relatively frequent occurrence received less attention in the early decades of the 20th century than did the more dramatic diseases caused by other pyogenic cocci Interest in staphylococci was stimulated considerably as a result of the Bundaberg disaster (Report of the Royal Commission 1928) Bundaberg a city in Australia was threatened with a diphtheria epidemic and toxin antitoxin mixtures were administered for prophylaxis One vial kept at room temperature in the summer heat without a preservative was used to inoculate 21 children Within several hours 16 of them became acutely ill and 12 died within 2 days with symptoms of septicemia or toxemia *Staph aureus* was recovered both from the vial and from lesions

Studies on the toxic effects of staphylococci and their products in animals led to the proposal that α hemolysin present in culture filtrates was responsible for lethality as well as dermonecrosis (Burnet 1929) For a time the clinical use of staphylococcal toxoid and antitoxin was in vogue but results were disappointing

The inability to incriminate a single product component or property of *Staph aureus* as the determinant of pathogenicity is a recurring feature in the history of research on staphylococcal disease Included in the list of substances or characteristics which have been advanced as primary factors are coagulase α toxin and hemolysins leukocidin lipase hyaluronidase and other enzymes and the ability of the organisms to resist phagocytosis or to multiply within phagocytic cells In each instance proponents have been unable to gather sufficient evidence to establish the role of the proposed mechanism Only the ability of staphylococcal enterotoxin to induce food poisoning but not invasive disease remains intact in the legacy of this search

In 1928 Alexander Fleming contracted to write on The Staphylococci for a systematic treatise on bacteriology Requiring more information on the variation of *Staph aureus* agar cultures he restudied the problem During this investigation a phenomenon was noted which was described in the text of the treatise (1929) as follows A common laboratory contaminant a *Penicillium* has a very marked lytic action on staphylococci growing in its neighborhood It is one of the ironies in the history of bacterial disease that the organism on which the effect of penicillin was originally seen had the capacity to produce an enzyme capable of destroying penicillin Over the course of time penicillinase producing penicillin resistant *Staph aureus* strains have become responsible for a significant proportion of staphylococcal disease The same phenomenon has occurred after the introduction of other antibiotics—fully pathogenic resistant strains have replaced susceptible strains of *Staph aureus*

Morphology

In stained preparations of pus *Staph aureus* appears as irregular groups of cocci

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17

Staphylococci and Other Micrococci

STAPHYLOCOCCUS AUREUS

Staphylococci are facultatively anaerobic, nonmotile gram positive cocci that tend to grow in irregular clusters Two species *Staph aureus* and *Staph epidermidis*, are currently recognized and they constitute a genus in the family Micrococcaceae (Breed *et al* 1957) *Staph aureus (Staph pyogenes M pyogenes* var *aureus)* is responsible for a variety of clinical disorders in man and animals ranging from pustules to food poisoning to fulminating septicemia However *Staph aureus* is also encountered as a normal inhabitant of human skin and mucous membranes in the absence of overt disease Although most strains produce a number of biologically active toxins and enzymes the mechanisms underlying the development and the persistence of disease are poorly understood Interest in the pathogenesis of staphylococcal disease has been aroused in recent years by an apparently increasing incidence of severe illness particularly in hospital environments where many strains are insensitive to the antimicrobial agents which have so successfully curbed the morbidity and the mortality of disease caused by other pyogenic cocci

History

In the latter half of the 19th century spherical microorganisms were repeatedly observed in suppurative lesions Bilroth (1874) recognized the diverse arrangement of the cocci and classified them as monococcus, diplococcus streptococcus and gliacoccus He postulated that all were forms of one organism, *Coccobacteria septica* and that their presence in pus was a secondary event and not of etiologic significance Koch (1878) found this view untenable and demonstrated at least 6 different disease syndromes in experimental animals injected with purulent material In each instance organisms identical with those originally present were recovered from the experimental lesions Koch s thesis regarding the induction of specific disease by specific microorganisms was substantiated with respect to abscess formation by both Pasteur (1880) and Ogston (1881)

Pasteur noted the presence of small spherical units, sometimes in pairs rarely in tetrads but most usually associated in small masses in pus from furuncles He also found entirely similar organisms in the contents of an osteomyelitic lesion leading him to state that osteomyelitis is a furuncle of the osseous medulla Studies in experimental animals supported the contention that both superficial and deep seated disease could be produced by the furunculosis organism

Ogston examined aspirated fluid from 82 abscesses and found cocci in all but 13, these 13 were cold abscesses Injection of pus containing the cocci produced either septicemia or localized suppuration in experimental animals Ogston noted that the cocci sometimes were found in chains whereas on other occasions clumps of cocci were seen Because of their aggregation he called the latter

species of blood used and the organism ex amined Rabbit blood is usually employed because erythrocytes of that animal are mark edly sensitive to the α hemolysin produced by most strains of *Staph aureus* isolated from human infections

Variation

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L forms have also been isolated from staphylococci grown in penicillin They dif fer from G colonies morphologically in their requirement for hypertonic conditions for initial growth and also in their ability to grow well under anaerobic conditions (Matt man *et al* 1961 Marston 1961a b) Sub cultures of L forms can grow in low salt con centrations and in the absence of penicillin for a number of weeks before reversion oc curs Like G colonies the L forms are non hemolytic and insusceptible to bacteriophage lysis coagulase production is also diminished

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The type strain of *Staph aureus* (Cowan *et al* 1954) liquefies gelatin in 24 hours at 27 C Milk is acidified at 37° C in 1 day with clot formation and liquefied and di gested at 5 and 7 days respectively The methyl red test is positive Acetoin is pro duced from glucose (positive Voges Pros kauer test) nitrates are reduced to nitrites catalase urease and phosphatase activities are found acid is formed from glucose xylose lactose sucrose maltose glycerol and mannitol whereas neither dulcitol nor salicin is fermented Again individual *Staph aureus* strains vary considerably from the type strain in their range of biochemical and enzymatic activities

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The individual cocci are quite uniform in size 0 8 to 1 0 micron in diameter and in general are smaller than the saprophytic micrococci Neither flagellae nor spores are present The cocci are spherical but their opposing surfaces may appear to be somewhat flattened

Staph aureus is strongly gram positive but individual cells in old cultures may lose the ability to retain the gram stain In infected material staphylococci ingested by phagocytic cells may be gram negative

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The fine structure of *Staph aureus* as revealed by electron microscopic examination is similar to that of most other gram positive organisms (Suganuma 1961) The finely granular cytoplasm is surrounded by a delicate membrane which is clearly separated from the thick rigid overlying cell wall Thus far morphologic substructure of the cell wall of *Staph aureus* has not been demonstrated

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Broth cultures are usually smooth but occasional strains yield a granular appearance After a few days the organisms begin to settle out and a scum often appears on the surface On storage of broth cultures in the refrigerator the cocci frequently form a viscid stringy mass

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Pigment production by *Staph aureus* varies from white to orange depending on the strain and the media used The golden yellow pigment which is most commonly found is due to the carotenoids δ-carotene and rubixanthene (Sabin and Stahly 1942 Suzue and Tanaka 1959) Enhanced pigment production is obtained by the addition of carbohydrates milk or glycerol monoacetate (Willis and Turner 1962) On blood agar plates colonies of *Staph aureus* frequently are surrounded by zones of clear hemolysis Since *Staph aureus* can produce 3 distinct hemolysins with different lytic spectra the extent of hemolysis depends both on the

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insoluble residue is the structured mucopeptide which confers spatial configuration to the bacterial cell In common with the mucopeptide of the cell walls of other gram positive organisms the mucopeptide of *Staph aureus* walls consists of a limited number of amino acids and the amino sugars N acetyl glucosamine and N acetylmuramic acid The amino acids characteristically present are alanine glutamic acid lysine and glycine

Antigenic specificity of the teichoic acid resides in the N acetylglucosamine moieties (Morse 1962c Nathenson and Strominger 1962) The amino sugar may be in either α or β glycosidic linkage and both configurations may confer immunologic specificity The heterogeneity requires that antisera utilized for the detection of the antigen recognize both configurations Polysaccharide A is a determinant of cell wall agglutination but it does not play a role in the agglutination of the intact organism It has been suggested that antibodies against *Staph aureus* cell wall teichoic acid i e Polysaccharide A are required for efficient phagocytosis (Mudd *et al* 1963)

An immediate skin reaction occurs in most adults after intradermal injection of Polysaccharide A fewer reactions are seen in children Low levels of precipitating antibodies are present in the majority of adult sera and higher levels are found in sera of patients with severe staphylococcal disease

Antigen A Another widely distributed antigenic component of *Staph aureus* is a protein termed Antigen A originally described by Verwey (1940) and further studied by Jensen (1958) and others (Lofkvist and Sjoquist 1962) Antigen A is a constituent of the cell wall and is released from the walls into solution by deoxyribonuclease (Yoshida *et al* 1963) It is a basic protein with a molecular weight of about 13 000 and it is destroyed by trypsin or chymotrypsin Antigen A is found in a majority of *Staph aureus* strains with the notable exception of the Wood 46 strain used in the commercial production of toxoid and is an important factor in the agglutination of intact bacterial cells Absorption of agglutinating antisera with Antigen A significantly reduces the agglutinin titer against a variety of strains of *Staph aureus* and thus the antigen is of no taxonomic value (Lenhart *et al* 1963) Its biologic properties in vivo are unknown but impure preparations are toxic for the isolated guinea pig ileum The antigen although an agglutinogen apparently plays no role in the phagocytosis of *Staph aureus* Antibodies against Antigen A are invariably present in normal human sera but are not found in normal rabbit sera

Capsular Antigen Homogeneous capsular material has been isolated from culture supernatant fluids as well as from bacterial cells of the distinctive mucoid Smith strain commonly used for experimental purposes The antigen is found in only a few strains of *Staph aureus* which have in common with the Smith strain a mucoid colonial appearance inability to be typed by bacteriophage and absence of bound coagulase (Morse 1962a 1963) The major structural unit of the antigen is 2 aminoglucuronic acid variable amounts of amino acids are also present (Perkins 1963 Haskell and Hanessian 1963)

Immunologically reactive polysaccharide material has been obtained from the supernatant fluid of stored cultures of *Staph aureus* Chemical analysis suggests that this represents polyglycerophosphate released from within the cell rather than true capsular antigen (Wiley and Wonnacott 1962)

Polyglycerophosphate ("Intracellular" Glycerol Teichoic Acid) Polyglycerophosphate first identified as an antigenic component of streptococci is found in many gram positive organisms including the staphylococci (McCarty 1959) The exact localization of polyglycerophosphate is unclear but it is not found in the cell walls and hence is termed an intracellular glycerol teichoic acid The antigen readily diffuses from the bacterial cell and it or related compounds represents a heterophile erythrocyte sensitizing antigen found in the culture supernatant fluids of *Staph aureus* and other gram positive organisms (Gorzynski *et al* 1960) Both unsubstituted polyglycerophosphate and chains with D alanyl units in labile ester linkage have serologic activity *Staph aureus* also contains glycerol teichoic acids with sugar substituents but their serologic activity is unknown (RajBhandary and Baddiley 1962)

has proved to be useful in epidemiologic studies but the multiplicity of ill defined agglutinogens the presence of blocking antigens, antigenic overlap and difficulties in preparing proper antisera preclude general usefulness Moreover differences in agglutination reactions may reflect only quantitative rather than qualitative differences in antigenic composition (Stern and Elek 1957) Attempts to distinguish between *Staph aureus* strains by precipitin hemagglutination and complement fixation reactions have been unsuccessful

In the absence of precise and practical methods of serologic differentiation bacteriophage typing of *Staph aureus* is utilized extensively Since Fisk s (1942) initial studies, the usefulness of this method has been extended by the selection of bacteriophages with restricted host range and by standardization of technics (Blair and Williams 1961 Wentworth 1963) From the large number of known lytic phages only a basic working set is generally used for routine typing

Group I 29, 52, 52A 79 and 80

Group II 3A 3B 3C 55 and 71

Group III 6 7, 42E 47 53 54, 75 77 and 83A

Group IV 42D

Miscellaneous 81 and 87

The phages in each group have similar host ranges and a given culture of *Staph aureus* is often lysed by more than one phage in a group occasionally a strain is lysed by bacteriophages in more than one group The phages in each group tend to be serologically related The mechanism determining specific susceptibility to bacteriophage is uncertain but it does not appear to be due to different receptor substances (Rountree 1947 Morse, 1962b) It is of interest that there is a rough correlation between serologic typing and bacteriophage typing (Oeding and Williams 1958) Certain biologic characteristics are also correlated with phage type e g the prevalence of multiple antibiotic resistance in strains typed by Groups I and III phages (Knight and Holzer 1954, Barber and Burston 1955)

In practice a culture of *Staph aureus* is flooded or swabbed onto an agar plate, and after drying bacteriophage suspensions are applied as fine drops at suitable distances from each other After incubation for 18 hours at 30° to 32° C, the bacteriophage strains which produce definite lysis are recorded and the phage pattern assigned to the organism e g, an organism lysed by phages 52A, 79 and 80 is designated as phage type 52A/79/80 The dilution of bacteriophage applied is that which just fails to give confluent lysis of the propagating strain—the routine test dilution or RTD Ten to 30 per cent of *Staph aureus* strains are not typable at the RTD but some of these are lysed at higher phage concentrations

Bacteriophages also exert a profound influence on the biology of *Staph aureus* The vast majority of strains are lysogenic and the existence of prophage affects such diverse properties as susceptibility to lytic phage production of various enzymes and hemolysins and resistance to antibiotics Transduction of these properties can be achieved in vitro (Morse, 1959, Rountree 1949 Blair and Carr 1961, Morse and La Belle 1962 Pattee and Baldwin 1962)

Staph aureus strains can also be distinguished by differences in susceptibility to various antibiotics and the determination of antimicrobial sensitivity serves as a useful adjunct to bacteriophage or serologic typing

Antigenic Structure

Polysaccharide A (Cell Wall Teichoic Acid) A species specific polysaccharide was isolated from virulent staphylococci by Julianelle and Wieghard (1935, Wieghard and Julianelle 1935) This antigen called Polysaccharide A recently has been identified as a carbohydrate component of the cell wall of *Staph aureus* (Haukenes *et al* 1961 Mc Carty and Morse 1964) It belongs to the group of bacterial polyolphosphate polymers called teichoic acids and consists of a backbone chain of ribitol linked 1 5 by phosphodiester bridges to form polyribitol phosphate (Baddiley 1962) N acetylglucosamine is in glycosidic linkage to C_4 of each ribitol and D alanine is in labile ester linkage to C_2 or C_3 of the ribitol residues Like the C polysaccharide of Group A streptococci Polysaccharide A is localized to the cell wall where it is attached by primary chemical bonds The antigen is solubilized by extracting the walls with trichloroacetic acid The

affects human as well as rabbit white blood cells leukocytes of other species are not susceptible Addition of leukocidin to suspensions of human granulocytes in the presence of calcium ions results in degranulation of the cells and the formation of cytoplasmic vesicles (Woodin *et al* 1963) Woodin (1959) found that P V leukocidin consists of 2 protein components both of which are required for leukotoxicity Antibody to either component protects against leukocyte damage (Gladstone *et al* 1962)

Free Coagulase Plasmas of many animal species are readily clotted by the extracellular or free coagulase of *Staph aureus* Free coagulase does not act directly to convert fibrinogen to fibrin but rather interacts with a plasma factor (coagulase reacting factor or CRF) to produce an active principle very similar to thrombin Both thrombin and the coagulase CRF principle release similar peptides from fibrinogen during clotting (Drummond and Tager 1963) Both have esterase activity (Haughton and Duthie 1959) and the esterase as well as the clotting properties are inhibited by diisopropylfluorophosphate but not by soybean trypsin inhibitor (Drummond and Tager 1962) However the coagulase-CRF factor differs from thrombin in not requiring calcium for its formation and in its relative insensitivity to heparin (Sanders 1963)

The plasmas of most animal species are susceptible to the action of free coagulase with the notable exceptions of the mouse and fowl which are deficient in CRF The action on guinea pig plasma is variable Studies on partially purified coagulase reveal it to be a heat labile protein which is destroyed by both trypsin and pepsin Less than 0 1 mcg will clot human plasma in 24 hours The intravenous injection of 22 mcg /kg is lethal for the rabbit, whereas 35 times that dose is required to kill mice (Duthie and Haughton 1958)

Coagulase is only weakly antigenic but at least 7 antigenically distinct staphylocoagulases have been recognized (Rammelkamp *et al* 1950 Duthie 1952 Zen Yoji *et al* 1961a b) There is a relationship between the type of coagulase produced by an organism and its bacteriophage type but no correlation exists with serologic classification

Bound Coagulase When *Staph aureus* cells are mixed with plasma on a glass slide thick clumps appear due to deposition of fibrin around the organisms The slide coagulase test correlates well with the tube coagulase test and originally it was believed that both reflected activity of the same product free coagulase It is now apparent that another coagulase bound coagulase is responsible for the positive slide test (Duthie 1954) Bound coagulase or clumping factor is not found in culture filtrates Antibody to bound coagulase prevents clumping of *Staph aureus* in plasma, and there is no inhibition by free coagulase antibodies Bound coagulase also differs from free coagulase in its spectrum of activity against various plasmas e g mouse plasma which is not efficiently clotted by free coagulase is readily coagulated by bound coagulase However the most significant difference between the two coagulases is that bound coagulase converts fibrinogen directly to fibrin without requiring an accessory plasma factor

Staphylokinase (Fibrinolysin) The fibrinolytic capacity of *Staph aureus* was recognized more than 50 years ago when it was noted that clots induced by staphylocoagulase lysed after several hours Lack (1948) demonstrated clearly that as in the case of streptococci clot dissolution by staphylococci is not the direct result of a bacterial fibrinolytic factor but is caused by activation of plasma plasminogen to the fibrinolytic enzyme plasmin For this reason the term staphylokinase is preferred to fibrinolysin Staphylokinase is formed by 70 to 90 per cent of strains and unlike streptokinase it activates plasminogen from many species and does not require plasma proactivator (Davidson 1960) Specific antibodies to staphylokinase inhibit its action on plasminogen (Quie and Wannamaker 1962) Staphylokinase is also responsible for the production of punctate areas of hemolysis and proteolysis which occur at a distance from colonies grown on blood agar the Muller phenomenon (Quie and Wannamaker 1961) Purified preparations of staphylokinase have not been obtained yet

Hyaluronidase Over 90 per cent of *Staph aureus* strains produce the enzyme hyaluronidase which depolymerizes mucopolysac

Other Antigens Immunodiffusion studies reveal that *Staph aureus* contains a multiplicity of other antigenic components which have not been isolated or characterized (Bernheimer and Schwartz 1961, Oeding and Haukenes 1963) Interestingly, antibody against one of these antigens is widely distributed in normal mammalian sera including that of germ free mice (Cohen *et al* 1963) Further studies may reveal a role for some of these antigens in pathogenicity as well as facilitating classification

TOXINS AND ENZYMES

α Toxin (α Hemolysin) Culture filtrates of many strains of *Staph aureus* lyse rabbit erythrocytes are lethal for experimental animals and produce local necrosis following intradermal injection It has long been suspected that one substance is responsible for these activities recent studies have confirmed this hypothesis (Bernheimer and Schwartz 1963, Lominski *et al* 1963) Purified α-toxin is a heat labile protein of molecular weight 44 000 As little as 0 008 mcg lyses a standard suspension of rabbit erythrocytes erythrocytes of sheep and humans are 25 and 150 times more resistant respectively Also 0 5 to 1 0 mcg of purified α toxin produces dermonecrosis and 4 0 mcg is lethal for the rabbit The precise mechanism of action of α toxin is not understood but it has no proteolytic activity Impure preparations cause marked contraction of smooth muscle followed by atony and flaccid paralysis striated muscle is not affected (Thal and Egner 1962)

Maximum amounts of α toxin are formed only after incubation for several days Yeast extract and increased carbon dioxide concentration appear to enhance production whereas anaerobiasis is inhibitory α Toxin is also formed in vivo The toxin is antigenic and toxoid is prepared by treating crude culture filtrates with formalin Anti α toxin blocks all of the biologic activity of the toxin

β Hemolysin, which is found more often in strains from animal sources is a hot cold hemolysin i e extensive lysis of erythrocytes does not occur during primary incubation at 37° C but does after subsequent storage at cold temperature (Glenny and Stevens 1935) Sheep ox and goat erythrocytes are markedly susceptible to β lysin whereas those of rabbit and man are resistant Divalent cations are required for hemolysis (Jackson 1963) Like α toxin β hemolysin is heat labile but production of the latter is not inhibited by anaerobiasis There is evidence that β lysin is a phospholipase which hydrolyzes sphingomyelin and that differences in the susceptibility of red blood cells from different species reflect the availability of this substrate (Doery *et al* 1963) β Hemolysin has less intrinsic toxicity for experimental animals than α toxin It is antigenic, and antitoxin prevents hemolytic activity

δ Hemolysin A third distinct hemolysin produced by *Staph aureus* is δ hemolysin (Williams and Harper 1947) which is considerably more heat stable in crude culture filtrates than α or β lysin Purified δ hemolysin is a trypsin sensitive protein with a molecular weight of approximately 70 000 (Yoshida 1963) It hemolyses human erythrocytes as well as those of the rabbit sheep erythrocytes are less susceptible Heated culture filtrates with δ hemolysin activity are not toxic but intravenous injection of concentrated material into rabbits is lethal (McLeod 1963) The most striking pathologic finding is an acute nephritic lesion The existence of a fourth hemolysin γ hemolysin has been postulated but many workers believe that it is identical with δ hemolysin (Smith and Price 1938 Elek and Levy 1950)

Leukocidins Three distinct leukocytotoxic substances can be found in culture filtrates of staphylococci The leukocidin described by Neisser and Wechsberg (1900) which agglomerates rabbit white cells and interferes with their respiration is identical with α toxin and has no effect on human leukocytes A thermostable leukocytotoxic substance termed leukolysin by Gladstone and van Heyningen (1957) causes marked morphologic changes in the leukocytes of all species examined except those of sheep Leukolysin is apparently identical with δ hemolysin The third leukocidin described by Panton and Valentine (1932) is not associated with hemolytic activity and is formed by approximately 40 to 50 per cent of strains The P V leukocidin or human leukocidin"

phosphatase (Barber *et al* 1951) and lipase and phospholipase (Magnuson *et al* 1962 Shah and Wilson 1963) Certain strains also produce an autolysin which differs from the lytic factor virolysin (Ralston *et al* 1957) produced during virulent phage infection

Ecology and Epidemiology

Staph aureus frequently is found in large numbers on normal human skin and mucous membranes and yet serious staphylococcal disease is comparatively rare Colonization begins in infancy within the first week to 10 days of life as many as 90 per cent of new borns are nasal carriers of *Staph aureus* Infants born at home acquire *Staph aureus* at a rate only slightly less than that found in hospital nurseries The carrier rate falls to about 20 per cent during the first 2 years of life but by age 4 to 6 it approaches the adult rate of 30 to 50 per cent (Williams 1963) The organism is found most com monly in the anterior nares and this is the site usually cultured in epidemiologic studies However significant skin carriage also oc curs particularly on the dorsum of the hands Usually the same phage type of *Staph aureus* is found both in the nares and on the skin of an individual carrier *Staph aureus* can also be isolated from throat cultures of asympto matic persons and intestinal carriage is esti mated at 20 to 30 per cent.

With respect to nasal carriage 3 kinds of individuals are recognized. Some 10 to 20 per cent of normal adults yield *Staph aureus* from the nose at all examinations and most keep the same phage type for many months or years (Roodyn 1960) An equal number of persons rarely harbor *Staph aureus* The majority of people are intermittent carriers That is they carry the organism for a period of a few weeks then are free from *Staph aureus* for a similar period and then once again are carriers often with a strain of a different phage type

The dynamics of acquisition are not fully understood but several important phe nomena have been observed As indicated the newborn rapidly acquires *Staph aureus* and in nurseries the strain carried is that predominant within the hospital unit (Barber *et al* 1953) The site of primary coloniza tion of the newborn is the umbilicus and the nasal carrier rate can be diminished markedly by maintaining sterility of the umbilical stump (Gluck and Wood 1961) Spread of the organism in the nursery is primarily by direct contact with personnel although it is well known that *Staph aureus* survives in fomites for long periods of time (Mortimer *et al* 1962)

Epidemics of suppurative disease in new born nurseries which are of considerable concern in many areas are usually associated only with certain strains of *Staph aureus* These strains designated hospital or "epi demic" strains are usually resistant to a variety of antibiotics Strains of the 80/81 complex became widely distributed in hos pitals throughout the world beginning in 1954 and were responsible for many nursery and other nosocomial epidemics other epi demic types usually in phage Groups I or III are also known (Parker and Jevons 1963) Even in the case of these epidemic strains healthy carriage among hospital personnel and patients is more frequent than is the occurrence of disease

In a nursery epidemic of staphylococcal disease as many as 50 per cent of infants will develop either superficial or deep sup-purative lesions (Shaffer *et al* 1957) The onset of disease may take place after dis charge from the nursery and it is not uncom mon for an infant to disseminate the hospital strain within the home and for lesions to de velop in family members particularly breast abscesses in nursing mothers (Hurst and Grossman 1960)

The acquisition and the carriage of *Staph aureus* by adults is a complex situation It is not known why certain persons are always carriers and why others are almost never carriers Moreover it is unclear why it is rare for a person to harbor more than one phage type of organism Most of the studies on the dynamics of the carrier state in adults have also been performed in hospital settings Ap proximately 20 to 30 per cent of patients admitted to hospitals will become carriers of the prevalent hospital strain the incidence of colonization increases with the duration of hospital stay (Noble *et al* 1964) Per sons who are already carriers have a lower acquisition rate than do those free of staph ylococci (Williams *et al* 1959) Acquisition

charides (Rogers, 1954) Hyaluronidase is antigenic and immunologic analyses thus far have revealed only one such enzyme in staphylococci

Penicillinase Many strains of *Staph aureus* particularly those lysed by bacteriophages in Groups I and III are resistant to penicillin Resistance is due to inactivation of penicillin by an enzyme penicillinase, which opens the β lactam ring of the penicillin molecule (Pollock, 1962) Penicillinase is a genetically constitutive enzyme but phenotypic expression i e the amount of enzyme formed is enhanced by the presence of the penicillin substrate Penicillinase production can also be increased by exposure of the organisms to some of the synthetic penicillins despite the fact that these compounds are only minimally susceptible to the enzyme (Batchelor *et al* 1963) Penicillinase formation has been demonstrated in strains isolated before the general use of the antibiotic suggesting that the current prevalence of penicillin resistant strains is the result of selection rather than recent mutation (Kirby 1944) Attempts to induce penicillinase formation in strains lacking the enzyme by exposing them to penicillin have been uniformly unsuccessful Penicillin resistance may develop in some strains treated in this manner but a different mechanism of resistance is apparently involved Moreover such strains in contradistinction to penicillinase producing organisms are unstable and nonpathogenic (Barber 1962) However penicillinase production can be induced in strains lacking the enzyme by transduction (Ritz and Baldwin 1961) Penicillinase producing *Staph aureus* may impair locally the action of *penicillin on other pathogens* (*Simon and* Sakai 1963)

Penicillinase may be found as either an endocellular or an exocellular product depending on the strain and the cultural conditions Purified exocellular penicillinase has been prepared recently (Richmond 1963)

Enterotoxin The majority of cases of food poisoning related to bacteria or bacterial products are caused by staphylococcal enterotoxin (Dack 1956) Enterotoxin is produced by only a few strains of *Staph aureus* and these are generally of phage type 6/47 or 42D (Allison 1949) However, the converse is not true and most organisms of these phage types do not produce enterotoxin Enterotoxin producing strains do not differ fundamentally from conventional strains of *Staph aureus* in terms of biochemical reactions or in the production of hemolysins coagulase, etc Therefore, identification of toxic strains depends on the direct demonstration of enterotoxin in culture filtrates Few experimental animals are susceptible to enterotoxin the most reliable species for testing are the monkey (*Macaca mulatta*) and man Vomiting can be induced in monkeys by the feeding or the intravenous injection of culture filtrates which contain enterotoxin in both instances there is a latent period of 1½ to 3 hours Several antigenically distinct types of enterotoxin exist A and B are the most common some strains produce both types (Casman *et al* 1963) The enterotoxins are trypsin resistant basic proteins rich in lysine which withstand boiling for 30 minutes Enterotoxin B has a molecular weight of 24 000 and is free of carbohydrate and lipid (Bergdoll *et al* 1959a Hibnick and Bergdoll, 1959) Intravenous injection of 1 mcg /Kg of purified toxin into monkeys produces emesis After an oral or an intravenous dose the animals become refractory to a second dose for a period of several days and this temporary resistance is specific for each antigenic type (Sugiyama *et al* 1962) Following repeated administration of enterotoxin a more lasting resistance develops which may represent true humoral immunity specific antibodies are known to block completely the effects of enterotoxin (Bergdoll *et al* 1959b) Enterotoxin is pyrogenic and its emetic action is enhanced by the prior administration of Thorotrast (Sugiyama *et al* 1963) The site of action of enterotoxin is not certain but the weight of evidence indicates that its effects are mediated by peripheral and/or central neurons rather than through a direct toxic action on the intestine (Clark *et al* 1962)

Other Substances Most strains of *Staph aureus* produce a number of enzymes capable of hydrolyzing substances in mammalian tissues or fluids These include nucleases (Cunningham *et al* 1960 Alexander *et al* 1961) proteases (Lack and Wailing 1954),

Dissemination from cutaneous lesions occurs by extension or embolization of infected thrombi and fortunately is rare Particularly dangerous are furuncles on the upper lip or in the nares because of the direct connection of the rich venous plexuses with the cavernous sinus Retrograde extension from such lesions is a common cause of cavernous sinus thrombosis Hematogenous spread of *Staph aureus* may lead to visceral abscess formation particularly in the kidney though indeed any organ may be involved

Acute hematogenous osteomyelitis a disease of young persons is most often caused by *Staph aureus* The incidence has decreased markedly during the antibiotic era Osteomyelitis presents with acute pain tenderness and swelling over the metaphysis of the long bones Early in the course roentgenograms of the bone are normal but then areas of medullary necrosis appear together with marked osteoblastic and osteoplastic reaction The organism can be recovered either from the lesion itself the periosteum or the overlying subcutaneous tissue Necrotic bone (sequestrum) may separate from viable bone and serve as a nidus for continued bacterial growth

Blood borne *Staph aureus* can induce a lesion on a heart valve which is congenitally deformed one previously damaged by rheumatic fever or occasionally on a normal valve The clinical outlook is grave and the rapid downhill course with valve destruction and metastatic abscess formation constitutes the most common form of acute bacterial endocarditis

Staphylococcal bacteremia is a state characterized by the almost constant presence of large numbers of *Staph aureus* in the blood stream Metastatic abscesses are numerous and each in turn may seed the bloodstream and continue the cycle The patient is acutely ill febrile and exhibits profound toxemia This syndrome was almost uniformly fatal before the introduction of antibiotic therapy unfortunately the mortality is still high (30 to 50%) Less hectic and more chronic staphylococcal bacteremia is also encountered and the source of seeding may not be apparent

Staphylococcal pneumonia in adults is almost invariably preceded by viral influenza and is one of the major causes of death during influenza epidemics and pandemics Patchy bronchopneumonia rather than lobar pneumonia is usual and the necrotizing lesions often progress to cavitary abscesses Empyema is common The onset is acute with high fever cough productive of purulent pinkish sputum and cyanosis fatalities are frequent *Staph aureus* pneumonia in children may occur as a complication of measles or whooping cough as well as influenza Recurrent bronchopneumonia is also a serious problem in children with cystic fibrosis In addition what appears to be primary staphylococcal pneumonia with extensive necrosis of pulmonary tissue is seen in infants particularly those under 1 year of age Empyema pneumatoceles and/or pyopneumothorax occur in more than 50 per cent of cases Cutaneous lesions in infants are less well localized than in adults and *Staph aureus* is a major cause of impetigo contagiosa *Staph aureus* is also one of the most common causative agents of neonatal meningitis

Staphylococcal pseudomembranous enterocolitis is characterized by copious diarrhea with attendant symptoms and signs of dehydration fever nausea abdominal pain and vomiting If the dehydration is extreme shock ensues Large numbers of *Staph aureus* are present in the diarrheal stools but even in their absence or in the presence of only low numbers of *Staph aureus* some workers feel that the organism has an etiologic role Many of the strains isolated produce both enterotoxin A and B (Warren *et al* 1963) This disease was clearly recognized before the antimicrobial era but became more frequent when persons were given antimicrobials which affected indigenous intestinal flora but not *Staph aureus*

One of the most troublesome features of invasive staphylococcal disease is the tendency for recurrence in the face of what appears to be an effective cure Osteomyelitis for example may recur as long as 20 years after the initial episode Exacerbations of disease generally are due to the same strain that caused the initial lesion It is believed the recrudescence is due to multiplication of small numbers of organisms persisters localized in necrotic foci or small abscesses that are protected from the host s cellular and humoral defense mechanisms as well as

of the hospital strains is enhanced in patients treated with antibiotics to which the hospital strain is insensitive (Knight *et al*, 1958) As with newborns the most important route of spread is probably direct contact but airborne dissemination and contamination of bed clothing may also play a role (Hare, 1963)

Outbreaks of *Staph aureus* disease occur in adult medical and surgical wards as well as in newborn nurseries The persons most susceptible are those who have undergone major operative procedures and those with severe underlying disorders including disseminated malignancy or leukemia, diabetes hyperadrenalcorticism agammaglobulinemia aplastic anemia or agranulocytosis and viral influenza (Nahmias and Eickhoff 1961)

The finding of asymptomatic persons who carry epidemic strains of *Staph aureus* has led to the controversial concept of the dangerous carrier However disease is not exclusively due to these organisms and many persons carry the epidemic strains without apparent harm to themselves or their associates Nevertheless the existence of strains with epidemiologic virulence calls for alertness to the possibility of outbreaks of serious disease

Both nasal and skin carriage of *Staph aureus* often are eliminated temporarily by topical application of antibiotics to the anterior nares This suggests that in many instances skin isolates represent transient contamination by organisms in the nose The carrier state can also be eradicated by systemic antimicrobial therapy but organisms of the original phage type often reappear 1 to 4 weeks after the cessation of treatment The mechanisms involved in the persistence of organisms are unknown

In view of the extremely high rate of carriage in human populations it is clear that the primary reservoir of *Staph aureus* is man but many domestic animals including dogs can be asymptomatic carriers of strains associated with human disease In addition strains implicated in diseases of animals such as those producing bovine mastitis may also produce disease in man

Diseases of Man

The characteristic lesion produced in man by *Staph aureus* is the abscess and the most frequent area of involvement is the skin Large numbers of granulocytes rapidly migrate to the site of bacterial localization and there is exudation of plasma components as well If the cocci are not ingested and destroyed during the initial inflammatory response more phagocytic cells and fluid accumulate In the fully developed lesion there is a central necrotic core filled with dead leukocytes and bacteria which is demarcated from surrounding tissue by a fibroblastic wall containing viable cocci and phagocytic cells As the necrotic material increases and liquefies the tension within the abscess rises accompanied by marked local pain and tenderness the abscess becomes frankly fluctuant and there is thinning of the overlying skin In the absence of therapy the abscess spontaneously bursts drains and heals Cellulitis and lymphadenitis occur if the lesion is not efficiently walled off Constitutional symptoms do not accompany small abscesses but with severe, deep seated lesions there is fever rapid pulse and a polymorphonuclear leukocytosis

Minor cutaneous abscesses are termed pustules and larger ones furuncles (boils) A more serious skin lesion is the carbuncle which is a laterally burrowing interconnected lesion usually occurring in the thick collagenous tissue of the back of the neck, that has multiple channels of egress to the skin Approximately 80 per cent of persons will incur one or more episodes of superficial lesions during their lifetime and the yearly incidence is somewhere between 2 and 10 per cent of the population Fortunately the lesions are usually self limited and not of serious consequence Nevertheless local abscesses, such as infected hangnails (paronychia) prevent normal function of manual tasks and may mean temporary economic disability Moreover 20 per cent of patients with one episode have one or more recurrences during the ensuing year and the risk of disease for members of the family is 4 times greater than in the general population (Kay 1962) A small number of patients have chronic recurrent furunculosis lasting over several months or even years due to the same phage type of *Staph aureus* It is impossible to predict which patients will fall into this category nor is it known why the episodes suddenly cease

that of mice Animals inoculated intravenously with 10^9 to 10^{10} organisms usually die within 24 hours whereas after an inoculum of 10^8 organisms the deaths occur after several days and suppurative renal lesions are the most striking finding (Cowan 1939b Stamp 1961 Li and Kapral 1962)

It is clear from the above that the experimental animals commonly used in the study of staphylococcal disease are markedly resistant to *Staph aureus* Other animals such as guinea pigs afford no particular advantage and although infection of the chick embryo has been studied this model is clearly unrelated to human disease The chinchilla is uniquely susceptible to staphylococcal enterocolitis (Wood *et al* 1956)

Many artifices have been designed to enhance the pathogenicity of *Staph aureus* in experimental animals The lethal intraperitoneal dose in mice is decreased markedly by suspending the organisms in mucin cutaneous lesions are more readily induced after tissue injury (Goshi *et al* 1961) systemic *Staph aureus* disease is enhanced by the administration of a variety of substances including thyroxine and dinitrophenol and animals are more susceptible during acute starvation (Smith and Dubos 1956b c) Resistance to staphylococcal disease is also increased by many nonspecific factors particularly after heterologous immunization (Cowan 1939b)

A few experimental studies have been performed in man Elek and Conen (1957) showed that the skin of normal humans is highly resistant to induced staphylococcal disease Formation of self limited pustules occurs only when more than 5×10^6 cocci are injected irrespective of whether the organism is isolated from normal flora or from lesions In surgical patients stitch abscesses are the most common form of staphylococcal disease the minimum pus forming dose in normal skin can be reduced to as few as 100 cocci if the organisms are impregnated on a silk suture tied through the skin The suture technic for enhancing *Staph aureus* infection is also applicable to experimental animals (James and MacLeod, 1961)

It is difficult to establish the carrier state for prolonged time periods in experimental animals but some information on the phenomenon of persistence has been obtained Following intracerebral or subcutaneous injection of *Staph aureus* organisms can be recovered for several weeks in the absence of a discernible lesion (Fisher 1962 Fisher and Robson 1963) Moreover despite prolonged intensive streptomycin therapy a few surviving fully streptomycin-susceptible *Staph aureus* cells can be found in the kidneys of intravenously injected mice for long periods of time (McCune *et al* 1956) Guinea pigs exposed to tetracycline resistant *Staph aureus* by aerosol retain culturable organisms in their nares for only a few days but the organisms can be evoked by the administration of tetracycline as long as 6 months after initial exposure (Simon 1963)

Pathogenicity and Virulence

The roles in the production of disease played by the many toxins and enzymes synthesized by *Staph aureus* are uncertain Local or systemic injection of culture filtrates containing α toxin into susceptible animals causes tissue necrosis or death (Blair 1958) but preformed toxin present in the usual bacterial inoculum apparently plays no role in the development of lesions Organisms suspended in saline are no less virulent than those suspended in culture medium containing α toxin (Elek and Conen 1957) The small role of α-toxin in the development of progressive lesions is further demonstrated by the general failure of active or passive immunization against α toxin to protect against the development of progressive disease (Smith 1937 Elek, 1959 Foster 1963) However rabbits injected with toxoid are protected against pustule formation in traumatized skin, although not in normal skin (Goshi *et al* 1961b)

Under certain circumstances α toxin seems clearly to be of importance For example immunization with toxoid prevents acute death in experimental animals given large intravenous inocula of *Staph aureus* In contrast, such immunization does not affect the outcome of rapidly fatal intraperitoneal infections associated with the finding of large amounts of α toxin in the peritoneal fluid (Koenig *et al* 1962) The paradox may re

from antimicrobial agents There is also speculation that *Staph aureus* may persist in an altered state such as the G form which is not readily cultured and which resists a variety of bactericidal substances

Food poisoning due to *Staph aureus* is due to ingestion of preformed enterotoxin The symptoms usually begin 3 hours after ingestion, but the time of onset ranges from 1 to 6 hours The heralding sign is increased salivation, followed by nausea, vomiting abdominal pain and watery diarrhea Recovery is generally within 24 hours and death occurs only when dehydration is excessive, particularly in infants or the debilitated

Diseases of Animals

Staph aureus is found in lesions in a variety of wild and domestic animals and birds but the specific role of the organism is difficult to define since other microorganisms are often present However disease in sheep and cows particularly bovine mastitis may be due solely to *Staph aureus* approximately 70 per cent of dairy herds in the United States have staphylococcal mastitis (Courter and Galton 1962) The strains isolated from animals are more likely to be of phage type 42D and to produce β hemolysin than human strains However strains of a variety of phage types including 80/81 are also found Many different strains of *Staph aureus* can also be carried by animals free of disease

The presence of *Staph aureus* in domestic animals may affect man Enterotoxin producing strains can incite bovine mastitis and raw milk and its products have been responsible for several outbreaks of food poisoning *Staph aureus* carriage and/or invasive disease can also be exchanged between man and domestic animals The use of antibiotics in animal food like the use of antibiotics in human populations is accompanied by an increase in the number of antibiotic resistant strains which are carried Thus 93 per cent of *Staph aureus* strains isolated from pigs fed tetracycline were resistant to the drug whereas only 5 per cent of strains obtained from animals not fed the antibiotic were resistant (Smith and Crabb 1960)

Experimental Infection

Most laboratory animals are relatively resistent to infection with *Staph aureus* and none of the experimental models is an accurate reproduction of human disease Intracutaneous inoculation of as many as 10^7 organisms in the mouse or the rabbit produces only small self limited necropurulent lesions (Johnson *et al* 1961, Fisher 1962) Larger doses in the rabbit cause spreading erythematous necrotic lesions Intramuscular injection of *Staph aureus* in mice induces local edema and infiltration with inflammatory cells The intensity of the reaction as determined by the degree of swelling is sometimes used as a criterion of the virulence of a particular strain (Selbie and Simon 1952)

The dynamics of intraperitoneal infection in mice have been studied extensively Quite large inocula of most strains 5×10^8 to 5×10^9 organisms are required to produce lethal disease In most cases death occurs within 24 hours and is associated with the production of large amounts of α toxin in vivo (Cohn 1962, Koenig *et al*, 1962)

Intravenous injection of approximately 10^8 to 10^9 viable units kills mice within 24 hours The acute deaths are usually ascribed to the production of α toxin in vivo When between 10^7 and 10^8 organisms are inoculated delayed deaths occur The injected cocci are cleared rapidly from the blood stream and can be recovered primarily from the major organs of the reticuloendothelial system the spleen and the liver (Smith and Dubos 1956a Fisher 1962) Lesser numbers are present in the lungs and the kidneys The titers of organisms in the spleen and the liver gradually decrease until 2 weeks later virtually no cocci can be recovered whereas the number of organisms in the lungs remains relatively constant However there is a gradual increase in the bacterial population of the kidneys which may reach 10^9 to 10^{10} staphylococci and renal abscesses are found Quantitative studies indicate that at least 10^4 organisms must be extracted by the kidneys after challenge before progressive fatal lesions develop the anatomic site of multiplication is the cortex The fate of rabbits receiving intravenous *Staph aureus* is similar to

termining the outcome of encounters with *Staph aureus*

Studies on the interaction between *Staph aureus* and phagocytic cells reveal that both heat stable and heat labile factors are required for efficient ingestion (Cohn and Morse 1959 Rogers and Melly 1962) The heat stable substance is specific antibody whereas the labile factor is presumed to be a component of the complement system Normal rabbit serum does not contain *Staph aureus* opsonizing antibody and the organisms are not ingested in vitro by either polymorphonuclear leukocytes or macrophages unless antiserum is added (Cohn and Morse 1959 Mackaness 1960) However phagocytosis of *Staph aureus* by fixed or circulating cells in the intact rabbit may not depend on opsonic antibody since very large inocula are required to produce disease It is known that *Staph aureus* is ingested in the absence of antibody by the process of surface phagocytosis *Staph aureus* is readily ingested by human leukocytes suspended in human serum which unlike rabbit serum normally contains opsonizing antibodies

The dynamics of the phagocytosis of *Staph aureus* in vivo have been studied in the peritoneal cavity of the mouse where ingestion of the organism by mononuclear and polymorphonuclear leukocytes has been shown to be efficient It is not certain whether normal mouse sera contain opsonizing antibodies

Once ingested more than 95 per cent of *Staph aureus* cells are killed rapidly within human and rabbit leukocytes in vitro and within mouse leukocytes in vivo (Cohn and Morse 1959 Mackaness 1960 Rogers and Melly 1960 Cohn 1962) It is uncertain whether the existence of small numbers of viable intraleukocytic organisms is of significance in the production or the persistence of *Staph aureus* disease The mechanism of killing within granulocytes is probably related to the presence within these cells of the bactericidal basic protein phagocytin (Hirsch 1960) since *Staph aureus* is resistant to lysozyme as well as to the acid pH of the intraleukocytic environment

Apparent defects in phagocytosis of *Staph aureus* are observed when an inoculum is manifestly larger than the number of cells mobilized to the inflammatory site or when emigration of phagocytes is retarded by vascular compromise or by the administration of adrenal cortical hormones Under these circumstances *Staph aureus* multiplies extracellularly at a rapid rate producing a variety of toxins and enzymes and the lesions may become progressive Phagocytosis of *Staph aureus* by mouse peritoneal cells can be impaired by suspending the inoculum in mucin but in man the induction of cutaneous lesions is not affected

The opsonic determinants of conventional strains of *Staph aureus* are not completely known Some workers suggest that antibodies against Polysaccharide A are necessary for phagocytosis to occur (Mudd *et al* 1963) In the case of the unique mucoid strains such as the Smith strain the capsular material is an important determinant of phagocytosis (Morse 1962a) Absorption of antiserum by the isolated capsular antigen removes opsonizing antibodies and immunization of mice with this antigen produces protection against an intraperitoneal challenge with the mucoid strains suspended in mucin Immunization with the antigen does not protect against challenge with conventional strains in mucin or the Smith strain suspended in saline (Morse 1962a Fisher *et al* 1963) Strains of the Smith type are encountered infrequently are readily phagocytized by human leukocytes and apparently are not of exceptional virulence for man

There have been many attempts to induce antibacterial immunity to conventional strains of *Staph aureus* in experimental animals In the case of *Staph aureus* antibacterial immunity implies the production of factors which increase phagocytosis of the organism since serum bactericidal or bacteriostatic antibodies are nonexistent Experimental studies are plagued by two major problems Most animals including man have a high degree of resistance to staphylococcal disease and protective effects which are observed are often of doubtful significance Secondly resistance to *Staph aureus* in both man and experimental animals often can be increased by immunization with heterologous bacteria or bacterial products (Cowan 1939b)

Despite these inherent difficulties available evidence suggests that specific antibacterial immunity to conventional strains of

flect the production of α toxin in the peritoneal cavity in amounts exceeding the binding capacity of antitoxin It is of interest in this regard that α toxin production in vivo is greater than that in vitro (Gladstone and Glencross 1960 Cohn 1962)

The role of α toxin in human staphylococcal disease is not clear It does not affect human erythrocytes and leukocytes but is capable of damaging skin The intracutaneous injection of an amount of preformed toxin sufficient to produce large areas of redness and swelling does not potentiate the ability of a given inoculum to produce a pyogenic lesion (Elek and Conen 1957) Moreover, approximately 4 per cent of strains isolated from human lesions do not manufacture α toxin in vitro (Lack and Wailing 1954) On the other hand Kleiger and Blair (1940) have pointed out that the symptoms of acute staphylococcal toxemia mimic the manifestations which follow the intravenous injection of α toxin into experimental animals and it is likely that α toxin was responsible for some of the deaths in the Bundaberg disaster

Neither β hemolysin nor δ hemolysin have been shown to be responsible for particular manifestations or the development of human disease, but it is noteworthy that δ hemolysin is toxic for human leukocytes

Coagulase has also been implicated as a factor in pathogenicity It was originally suggested that the deposition of fibrin about the organisms due to either free or bound coagulase might inhibit the ability of phagocytic cells to engulf *Staph aureus* (Hale and Smith 1945) careful studies fail to support this notion (Rogers and Tompsett 1952) Although injection of large amounts of free coagulase produces widespread clotting in vivo extensive intravascular clotting does not accompany severe or fatal staphylococcal disease (Fisher 1936) Strains of *Staph aureus* varying in ability to clot guinea pig plasma do not vary in capacity to produce disease in these animals (Foster 1962) Moreover laboratory derived mutants lacking free and bound coagulase are as pathogenic for rabbits as the parent strain (Li and Kapral 1962) Coagulase may actually act in favor of the host by localizing lesions through the deposition of a fibrin barrier and it is of interest that infants and children who tend to have more spreading lesions have lower levels of coagulase reacting factor than adults (Rammelkamp and Lebovitz 1956) On the other hand mice and fowl which are deficient in CRF, are very resistant to *Staph aureus* disease Specific protection by immunization with preparations rich in Type I but not Type III coagulase in rabbits has been reported (Lominski *et al*, 1962), but in other experiments (Fisher, 1962) protection was not correlated with titers of circulating anticoagulase

The P-V leukocidin destroys human leukocytes and it is possible that this effect in vivo aids the initiation and the progression of disease There is an inverse correlation between the level of cord blood antileukocidin and the incidence of staphylococcal disease in the newborn (Johanovsky, 1959 Banfer 1962) Moreover immunization of pregnant women with leukocidin rich toxoid decreases the incidence of disease in both the newborn and mothers Whether these observations reflect correlative or nonspecific factors awaits the results of intensive studies now being pursued

Other potentially injurious enzymatic products such as hyaluronidase lipase etc have been implicated as determinants of pathogenicity but they do not appear to be primary factors (Elek 1959) Strains possessing all these factors may be found in healthy carriers but strains lacking one or more of the enzymes or toxins mentioned (with the exception of free coagulase which defines the species) have been isolated from human lesions

The ability of a microorganism to survive and multiply in the host can be as important for pathogenicity as the elaboration of toxic substances *Staph aureus* is capable of surviving environmental circumstances inimical to other microorganisms it is unusually resistant to acid mercuric ions heat, hypertonic sodium chloride phenol and drying *Staph aureus* also resists the action of serum bactericidins most strains grow readily in broth containing serum Other body fluids which contain fatty acids lethal for many bacteria do not inactivate *Staph aureus* and the majority of *Staph aureus* strains are also not susceptible to killing by lysozyme

The ability or the inability of host cellular elements to ingest and destroy microorganisms probably plays a central role in de

form free coagulase but the converse is not true Therefore strains that are negative by slide test which otherwise resemble *Staph aureus* should be tested for free coagulase

A variety of differential and selective media are used for the separation and the identification of *Staph aureus* Mannitol fermentation is demonstrated by an appropriate indicator in media containing the polyol free phenolphthalein is liberated by *Staph aureus* phosphatase in phenolphthalein phosphate agar (Barber and Kuper 1951) tellurite is reduced and the organism grows as black colonies on tellurite glycine agar (Zebovitz *et al* 1955) and opacity is produced on solid media containing egg yolk by the action of lipases (Graber *et al* 1958) Selective media incorporate factors such as mercuric ions which are inimical to the growth of other microorganisms (Moore 1960) Most strains of *Staph aureus* grow readily in high salt concentrations Solid nutrient media containing 7 5 per cent sodium chloride are the most useful of the selective media None of the differential media replaces the coagulase test for identification of *Staph aureus* and the use of selective media excludes the growth of other microorganisms which may be of clinical significance For these reasons these media are more useful in epidemiologic studies than in routine laboratory procedures

Susceptibility of a micrococcus to *Staph aureus* bacteriophage clearly identifies it as a member of the species but bacteriophage typing is an epidemiologic rather than a diagnostic tool Determination of antibiotic sensitivity is also more important for clinical and epidemiologic purposes than for diagnosis Epidemic strains generally display a high degree of multiple antibiotic resistance However in the laboratory these organisms can not be differentiated from other *Staph aureus* strains

The physician often asks if a *Staph aureus* isolated from a lesion or from body fluid is of etiologic significance or merely a contaminant The laboratory cannot answer the question In view of the widespread environmental distribution of *Staph aureus* each specimen must be obtained with meticulous precaution to avoid extraneous contamination

Since direct tests for the presence of enterotoxin by feeding of material to monkeys or man is impractical the finding of large numbers of culturable *Staph aureus* in suspected food is presumptive evidence that the organism is the responsible agent in cases of food poisoning However food which has been contaminated and then heated will yield no culturable organisms though the enterotoxin remains active

Treatment and Control

Treatment Most instances of staphylococcal disease are relatively minor cutaneous lesions pustules and furuncles The basic principle of therapy of these lesions is to promote adequate drainage Spontaneous drainage is facilitated by the application of moist hot compresses surgical incision may be necessary but is performed only when the lesion is fluctuant. Systemic antibiotic therapy is not generally warranted Skin care with germicidal baths lessens contamination and decreases the likelihood of auto infection The patient must be warned against manipulation of the lesions to prevent hematogenous spread Carbuncles because of their burrowing characteristic generally do not drain adequately and often both antimicrobial therapy and surgical drainage are required

Serious staphylococcal disease—bacteremia endocarditis pneumonia meningitis acute osteomyelitis etc.—calls for the prompt administration of systemic antimicrobial therapy in large doses and therapy often must be continued for a prolonged time to effect cure The surgical removal or drainage of suppurative foci is of great importance in the outcome of these conditions

When penicillin was first introduced 85 to 90 per cent of *Staph aureus* strains were sensitive to the antibiotic Although there is considerable variation more than 80 per cent of strains now responsible for serious hospital acquired staphylococcal disease are penicillin resistant and 20 to 40 per cent of community strains also are not susceptible In addition hospital strains are frequently resistant to other antibiotics such as tetracycline about 50 per cent chloramphenicol about 40 per cent and erythromycin about 40 per cent (Barber and Burston 1955 Rogers 1956 Finland *et al* 1960 Petersdorf *et al* 1960 Cohen *et al* 1962 Cheatle

Staph aureus can be induced in mice and rabbits after administration of cells or cell fractions (Stamp, 1961 Fisher, 1962 Koenig *et al*, 1962 Ekstedt, 1963) In most instances the degree of protection is small and incomplete Passive administration of anti bacterial serum similarly produces partial protection against experimental disease

The importance of allergy to *Staph aureus* in pathogenesis of disease is uncertain but Johanovsky (1958) and Johnson *et al* (1961) have indicated that delayed hyper sensitivity to staphylococcal components in creases susceptibility to experimental disease

As previously stated some strains of *Staph aureus* are epidemiologically more virulent than others but attempts to eluci date the specific factors involved have been unsuccessful and these strains do not induce experimental disease more readily than others

It is obvious that orthodox approaches have not provided a thesis that satisfactorily explains the induction or the development of progressive staphylococcal disease This is particularly true in man in whom the situa tion is compounded by the frequent occur rence of apparently fully pathogenic strains in the absence of illness and the presence in the sera of most normal adults of antibodies to many *Staph aureus* components Varia tions in serum titer of a variety of these anti bodies with the possible exception of anti human leukocidin are not closely correlated with disease One must conclude that in man *invasion of tissues by Staph aureus is* pre vented in most instances by the rapid mobili zation of natural host defense mechanisms reinforced by specific antibodies In the face of local or systemic abnormalities or in the case of the newborn of physiologic imma turity the organism multiplies at a rate which surpasses the ability of the host to destroy the inoculum A multiplicity of unknown fac tors—bacterial toxins or enzymes inactiva tion or inaccessibility of antibodies and phag ocytes etc —then may come into play Delineation of these interactions has proved to be extraordinarily difficult

Persistence of organisms is a phenomenon which relates to pathogenicity as well as to healthy carriage Despite prolonged therapy with an effective antimicrobial agent small numbers of organisms are frequently found in necrotic foci In some situations organisms cannot be cultured and yet disease due to the same phage type of *Staph aureus* recurs after a variable time period It is unknown whether the organisms exist in a site inac cessible to antimicrobials or cultural technics are inadequate or the cocci are in an altered form

Diagnosis

The presence of typical irregular clusters of gram positive cocci in purulent material suggests the presence of staphylococci or other micrococci Precise identification of *Staph aureus* requires laboratory isolation Hemolysis and golden yellow pigmentation of colonies of gram positive cocci in charac teristic aggregation point to the diagnosis as does the ability of the organism to ferment mannitol However, *Staph aureus* strains differ in the types of hemolysins produced and certain strains are nonhemolytic Fur thermore pigmentation as well as fermenta tive capacities vary At the same time other micrococci are hemolytic and can form orange-colored colonies which are difficult to distinguish from those of *Staph aureus*

By definition a strain which produces free coagulase is *Staph aureus* Strains isolated from clinical material that do not produce free coagulase are extremely rare The test for free coagulase is performed by adding 0 5 ml of a broth culture or a loopful of agar growth to 0 5 ml of a 1 5 dilution of citrated *human or rabbit plasma in* a serologic test tube The mixture is incubated at 37° C Definite clot formation usually occurs within 3 hours but may not take place for 18 hours Each plasma lot should be tested with a known coagulase positive strain to ensure that inhibitors or coagulase antibodies are not blocking the reaction

Because free and bound coagulase gen erally are found together the simple and con venient slide test for bound coagulase is often used as a routine screening procedure (Cadness Graves *et al* 1943) A bacterial suspension is mixed with a drop of plasma or fibrinogen solution on a microscope slide in a positive reaction clumping of the ba teria occurs within 30 seconds Virtually all strains that produce bound coagulase also

multiply readily should be kept refrigerated until use Many different foods have been found to be the source of food poisoning out breaks but bakery products particularly those with cream fillings gravies and certain meats are implicated most frequently There is no external evidence such as a change in the taste or the smell of food that indicates contamination with *Staph aureus* Entero toxin is heat stable and therefore heating of contaminated food will not destroy the toxin though viable organisms are killed

Immunity in Man

Sera of most normal adults contain anti bodies to a variety of components and prod ucts of *Staph aureus* including α toxin co agulase human leukocidin hyaluronidase staphylokinase Polysaccharide A and the agglutinogen Antigen A In addition human gamma globulin protects mice against in vasive staphylococcal disease (Fisher 1959) Antibodies to α toxin leukocidin staphy lokinase Antigen A and the mouse protec tive factor are known to cross the placental barrier Antibody titers in the infant decline during the first few months of life and then rise over the next few years presumably as the result of the antigenic stimulus derived from carriage of *Staph aureus* or minor le sions (Quie and Wannamaker 1964) No correlation between the titer of a particular antibody and resistance or susceptibility to disease has been discovered It is noteworthy that an increase in antibody titer does not occur regularly in patients with disease even when the illness is chronic (Lack and Towers 1962)

Despite the presence of antibodies in nor mal sera and the apparent inability of natu ral infection with *Staph aureus* to induce protection against recurrence there is still interest in active immunization as a way of increasing resistance particularly in patients with recurrent furunculosis Among the ma terials that have been used for immunization are toxoids prepared from culture filtrates rich in α toxin or leukocidin killed *Staph aureus* isolated either from the patient (au togenous vaccine) or from other sources and lysates Reports of successes are approxi mately as numerous as those of failures Some of the successes may be related to non specific factors Studies on active immuniza tion continue in many parts of the world and as the components of the vaccines are eluci dated the materials standardized and spe cific serologic responses measured precise information may be forthcoming

STAPHYLOCOCCUS EPIDERMIDIS

Staph epidermidis (*Staph albus*) resem bles *Staph aureus* closely in microscopic morphology Colonies are almost invariably chalkwhite and zones of clear hemolysis due to ϵ hemolysin are seen on blood agar (Elek and Levy 1950) The organism grows well on most nutrient media biotin is required for aerobic growth and uracil for anaerobic growth (Jones *et al* 1963) Acetoin is formed from glucose and nitrates are re duced to nitrites A variety of sugars are fer mented but unlike *Staph aureus Staph epidermidis* does not ferment mannitol and gelatin is liquefied only at a slow rate The most important feature that differentiates the species is that *Staph epidermidis* strains do not produce coagulase they are often re ferred to as coagulase negative staphylococci Coagulase negative staphylococci are sensi tive to inhibition or killing by a variety of factors in the milieu such as high salt con centration which do not affect *Staph aureus*

The species antigen of *Staph epidermidis* is also a cell wall teichoic acid Polysaccha ride B which has a polyglycerophosphate backbone Monoglucoside units in α linkage which are the antigenic determinants are attached to some of the glycerols whereas others have D alanine residues in labile ester linkage (Morse 1963b) The antigen does not cross react with Polysaccharide A of *Staph aureus* but a common protein antigen in the two species has been reported (Elek 1959)

Staph epidermidis does not produce the extensive complement of toxins and enzymes found in *Staph aureus* and the organism is essentially nonpathogenic in experimental animals In man *Staph epidermidis* is a part of the normal mucocutaneous flora It pro duces disease less frequently than *Staph aureus* but may be responsible for minor cu taneous lesions such as infected acne and small pustules particularly stitch abscesses Serious disease including endocarditis and

1962) The recent synthesis of penicillins insusceptible to *Staph aureus* penicillinase, such as methicillin has materially aided therapy of disease due to penicillinase producing strains of *Staph aureus* (Batchelor *et al*, 1963)

Acute staphylococcal pneumonia or bacteremia is often a medical emergency, and penicillin plus methicillin is given until the sensitivity of the organism is determined Vancomycin is a useful drug in treating patients allergic to penicillin and some physicians utilize bacitracin and kanamycin despite their inherent toxicity *Staph aureus* strains that are resistant to methicillin have now been isolated and no doubt will increase in frequency The methicillin-resistant strains are fully capable of producing disease and the problem of antimicrobial therapy of *Staph aureus* disease is therefore by no means solved (Chabbert and Baudens 1962 Jevons *et al* 1962 Stewart and Holt 1963)

Other antibiotics such as erythromycin tetracycline and chloramphenicol are not indicated for the primary treatment of severe staphylococcal disease but may be of use in treating minor lesions and occasionally in combined therapy of disease which does not respond to the administration of a single antimicrobial agent

Passive administration of anti α toxin is not generally employed in the management of severe staphylococcal disease nor has the use of human gamma globulin as a specific therapeutic measure been rewarding. Both local and systemic bacteriophage therapy have been tried also without significant success

Control There is no practical way of removing *Staph aureus* from its widespread and usually benign association with man Efforts are directed toward the prevention and the control of disease in hospitals where contact occurs between susceptible persons and apparently more virulent strains Hospitals tend to have their own unique staphylococcal problem and the uncertainties regarding the spread and the acquisition of *Staph aureus* prevent the delineation of firm rules Certain general principles are of importance Persons with staphylococcal lesions disseminate large numbers of organisms and they should be segregated from adult susceptibles or newborns Similarly, ward personnel with open lesions are not allowed to perform their usual duties Highly susceptible individuals, such as those with agranulocytosis are protected from other patients and personnel who are carriers Especially to be avoided is the indiscriminate use of antimicrobials which may lead to the acquisition of resistant strains by the patient and the prevalence of such strains within an institution Finally all minor or major surgical procedures and wound care should be performed with maximal asepsis

Questions regarding the disposition of asymptomatic carriers of an epidemic strain are difficult to answer except in specific situations It is advisable that each institution establish a surveillance committee to evaluate the risks involved and to act promptly if an epidemic occurs Under epidemic conditions a search for the source of infection is initiated and personnel who are carriers of offending strains are removed from the ward and not permitted to return until the carrier state is eliminated (Nahmias and Eickhoff 1961 Williams and Shooter 1963) Nevertheles despite intensive efforts to eliminate carriers and isolate patients closed epidemics are often recurrent, and entire wards have had to be closed

The newborn is particularly susceptible to staphylococcal disease and decreased colonization by hospital strains is achieved by care of the umbilical stump and the skin with germicidal liquid or powder Shinefield and co workers (1963) recently presented a novel way of protecting infants from acquiring epidemic strains A penicillin sensitive strain of *Staph aureus* which does not have epidemiologic virulence is implanted in small numbers in the nares or on the umbilicus soon after birth The carrier state thereby established seems to prevent or retard colonization by epidemic strains during the limited hospital stay of the infant This is an example of a process variously termed interference infection immunity or premunition

Prevention of food poisoning due to *Staph aureus* involves avoidance both of food contamination and bacterial growth Persons with open lesions should not participate in the preparation of food More importantly foodstuffs in which enterotoxinogenic strains

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bacteremia, can also occur, particularly in debilitated persons and the mortality is high (16 to 20%) Therapeutic principles for *Staph aureus* disease apply also to *Staph epidermidis*—drainage of abscesses and administration of antimicrobials in severe disease

Many strains of *Staph epidermidis* are resistant to antibiotics In a recent study of 175 clinical isolates most of them from blood cultures 50 per cent were resistant to penicillin and 10 per cent were resistant to methicillin (Kjellander *et al*, 1963) Insusceptibility to penicillin is due both to the production of a penicillinase which like *Staph aureus* penicillinase is a beta lactamase and to a level of inherent resistance (Kjellander and Finland 1963) Many of the strains were also resistant to chloramphenicol tetracycline and erythromycin Thus the antimicrobial therapy of serious disease due to *Staph epidermidis* holds many of the problems found in treating illness caused by *Staph aureus*

OTHER MICROCOCCI

Gaffkya tetragena (M tetragenus) This *species of the genus Gaffkya is distinguished* by the arrangement of cells in packets of fours (tetrads) which are often surrounded by a capsule or pseudocapsule These features are found in material obtained directly from animal tissue or fluids but in artificial culture the characteristics may not be apparent *Gaffkya tetragena* is slower growing *than the staphylococci It is a facultative* anaerobe On agar, the colonies are white-to gray moist gleaming and viscid A variety of sugars are fermented but not mannitol Gelatin is not liquefied Coagulase is not formed by *Gaffkya tetragena* nor by the other micrococci The organism can be isolated from the normal upper respiratory tract or the skin of man and frequently is found in the sputum of tuberculous patients *Gaffkya tetragena* produces a rapidly fatal septicemia in mice In man either localized or septicemic disease occasionally occurs particularly in persons with lowered general resistance

Sarcina The cocci in this genus tend to divide perpendicularly in 3 planes resulting in the formation of packets of 8 16, 32 or more elements Some species are anaerobic but the species most commonly isolated from the environment of man is the aerobic *Sarcina lutea* *Sarcina lutea* produces sulfur to chrome yellow colonies on agar, and pigment is formed in liquid culture Sugars are not generally fermented Human disease caused by *Sarcina lutea* is extremely rare

The other aerobic species of micrococci which, like staphylococci, grow in irregular masses constitute a heterogeneous group included in the genus *Micrococcus* They are of relatively little importance in human disease

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18

The Meningococci

The meningococci are gram negative cocci, usually occurring in pairs which form non pigmented translucent colonies and ferment dextrose and maltose with production of acid They are aerobic nonsporulating and non motile They are strict parasites of man and are the cause of several types of infections of which the most important are meningo coccemia and meningococcal meningitis The accepted name is *Neisseria meningitidis* al though occasionally used synonyms are *Neis seria intracellularis* and *Diplococcus intra cellularis meningitidis*

HISTORY

Epidemic cerebrospinal meningitis is un doubtedly an ancient disease but its early history remains unclear because of the diffi culty in differentiating it from other syn dromes of a similar nature This confusion is exemplified by some of the names used to describe the disease in the early 19th century viz sinking typhus spotted fever and brain fever According to Hirsch (1886) the disease was first recognized in epidemic form in Geneva Switzerland in 1805 by Vieusseux and 1 year later in the United States at Medfield, Mass where an outbreak was described by Danielson and Mann Dur ing the next 75 years an increasing number of outbreaks of a disease which may be pre sumed to be epidemic cerebrospinal menin gitis on the basis of clinical and epidemiologic data occurred in Western Europe and the United States Military forces particularly those stationed in barracks seemed to be un duly prone to epidemics of the disease By the end of the century outbreaks had been re ported from Asia Africa and Australia as well The isolation and the description of the causative organism *Neisseria meningitidis* from spinal fluid of a clinical case by Weich selbaum in 1887 permitted the diagnosis to be made with certainty Since then extensive outbreaks have occurred in many parts of the world A major epidemic occurred in New York City in 1904 and 1905 During World War I the Allied Forces both in England and on the Continent, suffered from outbreaks of the disease In the United States in recent years it has tended to occur at irregular inter vals There was a severe outbreak in the period of 1928 to 1930 and another during the opening years of World War II 1940 to 1943

With the widespread application of diag nostic laboratory procedures our knowledge of the disease increased rapidly It was recog nized that meningococcal meningitis could occur endemically as well as in the form of epidemics A septicemic form of the disease in which the central nervous system was not involved was recognized and given the name meningococcemia The organism was iso lated from the nasopharynges of a significant number of individuals during the course of routine surveys and based on this evidence

the concept of the healthy carrier developed

Dopter (1909) was the first to recognize the existence of serologically specific types of meningococcus This work was continued and extended by British and French workers during World War I and laid the firm basis for our present system of classification Serum therapy for meningococcal meningitis was introduced by Jochmann in 1906 and its use was firmly established by the work of Flexner (1913) The era of chemotherapy started in 1939 and today this form of treatment has completely displaced serum therapy The outbreaks of epidemic disease which accompanied World War II gave investigators the opportunity to carry out valuable investigations on the epidemiology the therapy and specific prevention of the disease The recent reviews by Branham (1953) by Goeters (1954) and by Scherp (1955) may be consulted for further reference

MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS

The meningococci are gram negative nonmotile nonsporulating cocci approximately 0 6 to 0 8 microns in diameter which in body fluids and liquid media at least are often arranged in pairs giving rise to the synonym diplococcus When found in pairs the adjacent sides are usually flattened to produce the typical biscuit or reniform shape Considerable variation occurs in both size and staining properties particularly in older cultures Autolysis is a prominent characteristic of the meningococci and is presumably responsible for the numerous ghost cells seen in older cultures as well as for the marked variations in staining properties The organism is usually described as being nonencapsulated since no capsule has been demonstrated unequivocally by direct capsular stains On the other hand in at least 2 groups of meningococci a capsule may be demonstrated in freshly isolated strains by the application of homologous typing serum

On solid media meningococci give rise to smooth nonpigmented and nonhemolytic colonies Freshly isolated strains produce transparent glistening colonies with a smooth border when grown on a transparent medium The so-called lens effect may be observed in that when distant objects are viewed through the colony they become sharply focused Some strains tend to produce mucoid colonies As the colony ages it tends to become opaque and granular and to lose its smooth border Old laboratory strains frequently give rise to rough colonies

Ever since the publication of Murray s monograph (1929) meningococci have been regarded as highly fastidious organisms which require accessory growth factors such as are present in blood serum and certain vegetable extracts Many media incorporating one or more of these products have been devised for the propagation of these organisms However their definitive growth requirements remain to be determined and more recent work by Frantz (1942) among others suggests that the primary difficulty is not a nutritional deficiency but rather the extreme sensitivity of meningococci to the toxic effects exerted by a variety of amino acids fatty acids and salts The role of animal proteins and vegetable extracts in culture media appears to be one primarily of absorbing toxic substances so as to permit growth rather than the furnishing of any necessary growth requirement An atmosphere containing 5 to 10 per cent carbon dioxide enhances the growth of most strains of the organism

The meningococci grow best at a temperature of 35 to 37° C At temperatures higher or lower than this the amount of growth falls off rapidly The organisms are strict aerobes and no growth takes place under strictly anaerobic conditions Blood agar chocolate agar trypticase soy agar and the starch casein hydrolysate agar of Mueller and Hinton (1941) represent the solid media in common use for the propagation of these organisms Growth in broth (tryptose phosphate veal infusion starch casein hydrolysate) is relatively poor resulting in a granular turbidity and little or no surface growth

The carbohydrate fermentation properties of these organisms are sharply limited Dextrose and maltose are fermented with the production of acid but no gas Lactose sucrose levulose and other sugars are not fermented Indole and hydrogen sulfide are not formed but catalase is present In common with other organisms belonging to the genus *Neisseria* meningococci contain cytochrome

TABLE 1 GROUPING OF MENINGOCOCCI

RECOMMENDED BY SUBCOMMITTEE 1950	DESIGNATION IN COMMON USE 1940 1955	GORDON AND MURRAY (1915) TYPES
A	I	I III
B	II	II
C	II *alpha*	
D	IV	IV

oxidase which rapidly oxidizes dimethyl- or tetramethyl paraphenylene diamine hydrochloride (McLeod *et al* 1934) The presence of this oxidase cannot be demonstrated in killed organisms It provides a useful means for the provisional differentiation of the members of the genus *Neisseria* from other microbial organisms which with rare exceptions lack this enzyme

As previously mentioned, the possession by meningococcus of an active autolytic enzyme system results in swelling loss of staining properties and in the ultimate disappearance of the cell itself in cultures more than a few hours old This process may be halted by the inactivation of the autolytic enzymes either by heating the culture to 65° C for 30 minutes or by the addition of potassium cyanide or formalin

The resistance of the meningococci to physical and chemical agents is relatively low and these organisms experience considerable difficulty in surviving outside the human body They are quite susceptible to desiccation and are killed by heating to 55° C for 30 minutes or by exposure to any of the common germicides in relatively low dilutions Most strains of meningococci are sensitive to the action of the sulfonamides penicillin and broad spectrum antibiotics such as the members of the tetracycline series Although fresh strains are sensitive to the action of streptomycin highly resistant forms develop rapidly and in some instances streptomycin dependent strains are the end result

ANTIGENIC STRUCTURE

At least 4 broad serologic groups of meningococci have been recognized on the basis of antigenic differences The original classification of Gordon and Murray (1915) has been followed in its general outline but the use of different terminologies by English French and American workers has caused a considerable amount of confusion The subject has been reviewed by Branham (1953 1958) The classification described in Table I follows that recommended by a subcommittee of the Nomenclature Committee of the International Association of Microbiologists in 1950 For clarification the terminology in common use in the United States during the period *1940 to 1955* is set up in parallel together with the original classification of Gordon and Murray

Of the 4 types originally described by Gordon and Murray I corresponds to Group A II to Group B, and IV to Group D It is the general consensus today that their Type III cannot be distinguished from Type I and therefore both are included in Group A Strains falling in this group (A) are the causative agents of the great majority of epidemic outbreaks of the disease Group B has been responsible for the majority of sporadic cases occurring in interepidemic periods Group C originally designated II *alpha* has been isolated chiefly from sporadic cases of the disease Very few strains falling into Group D have been isolated in the United States There is relatively little crossing over between the 4 groups In addition to these a number of nasopharyngeal strains have been isolated which have not been agglutinated by any of the specific antisera These organisms apparently play no significant role in the causation of disease The production of group transformation has been described by Alexander and Redman (1953)

Group A and C strains are better antigens than those belonging to Group B and long courses of immunization are necessary with the latter in order to produce high titered diagnostic antisera Chemical fractionation (Scherp and Rake 1935 Kabat 1943) has revealed 3 types of antigenic substances A nucleoprotein or P substance is found in all meningococci as well as in other *Neisseria* and in Type III pneumococci This material apparently contributes to the toxicity of these organisms A polysaccharide or C substance is also found in other members of the *Neisseria* as well as in certain unrelated or

ganisms A Boivin type toxic glucolipid is present in all meningococci and is closely related to similar antigens found in other *Neisseria* In addition a polysaccharide specific for Group A meningococci has been identified and purified in the form of the sodium salt of a polysaccharide acid Although not a complete antigen in itself it is presumably responsible for the specific serologic reactions given by Group A Among these are included the capsular swelling and the so-called halo reaction This last reaction represents the formation of a ring of precipitate around the individual colonies of the Group A meningococci when they are inoculated into an agar medium containing an excess of specific antibody Similar reactions have been observed with Groups B and C strains when they are brought into contact with specific antisera In the case of Group C meningococci this reaction is due to a capsular polysaccharide haptene which is predominantly a polymer of a sialic acid congener (Watson *et al* 1958) No capsule has been demonstrated in Group B meningococci the specific surface haptene appears to be a noncapsular carbohydrate containing polypeptide In the case of Group A antisera at least there appears to be a close correlation between their antipolysaccharide content and their protective ability After absorption of the antiserum with the specific polysaccharide there remains some residual antibody which affords appreciable protection in mice against experimental meningococcal infections (Kabat *et al* 1945)

Group B meningococci contain a protein fraction which is common to all members of the group and is antigenic In addition there is a carbohydrate polypeptide complex (kappa substance) which although not a complete antigen in itself reacts with Group B specific antibodies

Since the early work of Flexner (1907) investigators have recognized the lethal activity of heat killed or autolyzed cultures of the meningococci The fact that such preparations are markedly toxic for laboratory animals has suggested that an endotoxin released by the autolysis of the bacteria is responsible for many of the signs of the disease The endotoxin appears to be relatively nonspecific antigenic and heat stable presumably it contains both nucleoprotein and glucolipid elements

NATURAL HABITAT AND RANGE OF PATHOGENICITY

The meningococci are strict parasites of man their natural habitat is the nasopharynx of this species Natural infections are found only in man and the organisms have a relatively low virulence for other species Flexner (1907) was able to produce a picture of meningitis in monkeys following the intraspinal inoculation of a relatively large number of organisms As pointed out by Murray (1929) laboratory animals such as mice guinea pigs and rabbits are susceptible to the inoculation of meningococci only when the infecting dose is so large that it approaches the lethal dose of the heat killed organisms However if the organisms are suspended in 2 to 5 per cent hog gastric mucin (Miller 1933) a relatively small number of organisms suffices to initiate a fatal infection The enhancing effect of the mucin is due presumably to the protective effect it exerts against the body s natural defense mechanisms Although this technic has been employed in the determination of antibody levels in sera and of the relative efficacy of drugs this type of infection bears little resemblance to the natural disease as it is seen in man hence its usefulness is limited. Embryonated eggs are susceptible to infection following inoculation of the organisms via the yolk sac route and this technic too has been employed for the assay of antibody levels and the therapeutic efficacy of certain drugs There is a considerable variation in virulence between strains of meningococci Old laboratory strains and those which have gone rough are of relatively low virulence as compared with strains freshly isolated from human beings

PATHOGENESIS

Meningococci gain entrance to the human body via the nasopharynx. After becoming implanted in this area they may set up a localized inflammatory reaction or they may remain completely quiescent giving rise to no signs or symptoms In a relatively small proportion of these infections invasion of the

bloodstream takes place This may remain limited as a simple bacteremia, or as the organisms are disseminated through the body, metastatic lesions may be set up in various sites such as the skin joints, ears lungs and adrenal glands and most important of all the central nervous system Here an inflammatory reaction involving the meninges of both the brain and the spinal cord is the chief finding While it is possible that in some instances organisms spread directly from the nasopharynx into the meninges by penetrating through the cribriform plate it is generally accepted that the central nervous system is invaded from the bloodstream Those factors which govern the ability of the organisms to spread from the nasopharynx into the bloodstream and ultimately to reach the central nervous system in a small percentage of these cases remain undetermined In most infections it appears that the organisms do not progress farther than the nasopharynx

It is not clear what are the mechanisms by which the meningococci give rise to the characteristic pathologic findings Presumably the endotoxic material released from the bacterial cells as a result of the process of autolysis is responsible for the initiation of the process The vascular system appears to be particularly sensitive to the action of this material and hemorrhagic manifestations (perhaps due to a Shwartzmanlike phenomenon) are common in the disease In fulminating meningococcemia this hemorrhagic process is particularly noticeable in the skin and in the adrenal cortex. The so-called Waterhouse Friderichsen syndrome is occasionally associated with massive adrenal hemorrhage but widespread vascular dysfunction causing peripheral pooling of the blood appears to be the usual cause of the profound shock associated with this condition rather than acute adrenal cortical failure

Meningococcal infection of the nasopharynx may set up so mild a reaction in the host that it attracts no notice In other instances an acute inflammatory process may progress to the stage of a purulent rhinitis

Meningococcemia presents the picture of acute sepsis with fever chills malaise and prostration Usually the typical rash can be detected early in the disease This consists of dusky red spots or petechiae of varying sizes up to 15 mm in diameter which involve the skin and the mucous membranes of the body In severe cases the petechiae may assume a purpuric appearance as the result of hemorrhage These may reach a diameter of several centimeters and go on to actual necrosis In mild cases the spots disappear within a few days leaving small brownish areas in their wake histologic examination has revealed that these lesions are due to thrombo-embolic involvement of the capillaries Puncture of the lesion frequently demonstrates the presence of meningococci Fulminant infections often give rise to the Waterhouse Friderichsen syndrome characterized by circulatory collapse and shock Diffuse hemorrhage into and necrosis of the adrenal glands are occasionally found at autopsy but these appear to be associated rather than essential pathologic lesions (Ferguson and Chapman 1948) In the absence of this complication recovery is the general rule if the disease is treated vigorously and promptly

Invasion of the central nervous system is marked by signs and symptoms of meningeal irritation and inflammation Severe headache pain in the posterior aspects of the neck on forward flexion nausea vomiting and often coma are prominent features Physical examination reveals muscular spasm stiff neck exaggerated reflexes and positive Kernig and Brudzinski signs Convulsions and bulging fontanelles are common findings in infants

The meningitis is characterized by an acute inflammatory reaction accompanied by thromboses of the smaller blood vessels and hemorrhage In the later stages marked thickening of the meningeal coverings is a prominent feature as the originally purulent exudate becomes organized Encephalitic involvement is characterized by focal areas of hemorrhage thrombosis and perivascular infiltration

IMMUNITY

The mechanism of recovery from clinically recognizable meningococcal infections has not been determined During the course of the clinical illness antibody formation may be demonstrated by a variety of technics including agglutination mouse protection and bactericidal tests among others The role that this antibody formed during the course of the

illness plays in the recovery of the patient remains uncertain Because of the low morbidity rate which makes it statistically improbable that second attacks will occur and because of the existence of several serologically specific meningococcal groups or types nothing is known concerning the potential immunity conferred by a clinical attack of the disease

There is no reliable method for measuring the susceptibility of an individual to either clinical or subclinical infections with meningococci Man appears to be relatively susceptible to inapparent nasopharyngeal infections with these organisms This is evidenced by the fact that a significant carrier rate of meningococci can be demonstrated in the general population throughout the year Most of these infections are entirely silent although a few may give rise to a local inflammatory process in the nasopharynx On the other hand the resistance of man to clinical infections appears to be relatively high in that cases of meningococcemia and meningococcal meningitis are relatively rare even during epidemic periods It has been shown by Thomas and Dingle (1943) among others that a good proportion of the adult human population possesses bactericidal antibodies against the meningococci which may be demonstrated in the test tube The role played by these protective antibodies in limiting the number and the severity of clinical infections remains undetermined However the erratic results obtained with various strains of meningococci suggest that it does not play a dominant role Presumably these antibodies are formed in most instances as a result of inapparent infections but this theory remains to be proved

Although there is some degree of positive correlation between the presence of group specific antibodies and the protective value of a given serum (Branham 1940) the fact that absorption with the whole homologous organism (as opposed to the specific polysaccharide alone) is necessary for the complete removal of the protective effect of the serum suggests that some other material (antigen or haptene) is playing a significant role in the production of protective antibody Presumably this protective antibody or antibodies exerts its beneficial effect through promoting phagocytosis by a process of opsonization and through enhancing the bacteriolytic process In this last regard it appears that both group specific antibody and complement play significant roles in the mechanism of the process Although numerous attempts have been made to develop a satisfactory process of active immunization the results in terms of protective antibody formation have been inconclusive and in general poor

DIAGNOSIS

The laboratory diagnosis of meningococcal infection is made by the isolation and the identification of the specific organism Blood spinal fluid and nasopharyngeal swabs represent the 3 most important materials to be examined for the presence of these organisms Although the technics required for the isolation and the identification of these organisms are not difficult they do necessitate careful attention to the details of the work Furthermore a thorough knowledge of the pathogenesis of the disease is required in order that cultures may be taken from proper sites at the correct moment

The importance of blood cultures in the diagnosis of meningococcemia is obvious in meningococcal meningitis these are positive in approximately half the cases if they are taken early in the course of the disease Blood taken from the basilic vein is inoculated into both liquid and solid media From 5 to 10 ml of blood is added to 100 ml of tryptose phosphate veal infusion or casein hydrolysate broth Approximately 0 1 ml of blood is spread over the surface of a blood agar chocolate agar or casein hydrolysate plate These are incubated at 35° to 37 C under conditions of increased humidity and carbon dioxide content (5 to 10%) Cultures should be inspected daily for 7 days before being discarded as negative In cases of overwhelming sepsis it is not uncommon to recognize gram negative diplococci in simple blood smears prepared for routine hematologic examination Similarly in such cases where petechiae or a purpuric rash are a prominent feature it is often possible to demonstrate the specific organism in these lesions by means of smear or culture or broth

bloodstream takes place This may remain limited as a simple bacteremia or, as the organisms are disseminated through the body metastatic lesions may be set up in various sites such as the skin, joints, ears lungs and adrenal glands and most important of all, the central nervous system Here an inflammatory reaction involving the meninges of both the brain and the spinal cord is the chief finding While it is possible that in some instances organisms spread directly from the nasopharynx into the meninges by penetrating through the cribriform plate, it is generally accepted that the central nervous system is invaded from the bloodstream Those factors which govern the ability of the organisms to spread from the nasopharynx into the bloodstream and ultimately to reach the central nervous system in a small percentage of these cases remain undetermined In most infections it appears that the organisms do not progress farther than the nasopharynx

It is not clear what are the mechanisms by which the meningococci give rise to the characteristic pathologic findings Presumably the endotoxic material released from the bacterial cells as a result of the process of autolysis is responsible for the initiation of the process The vascular system appears to be particularly sensitive to the action of this material and hemorrhagic manifestations (perhaps due to a Shwartzmanlike phenomenon) are common in the disease In fulminating meningococcemia this hemorrhagic process is particularly noticeable in the skin and in the adrenal cortex The so called Waterhouse Friderichsen syndrome is occasionally associated with massive adrenal hemorrhage but widespread vascular dysfunction causing peripheral pooling of the blood appears to be the usual cause of the profound shock associated with this condition rather than acute adrenal cortical failure

Meningococcal infection of the nasopharynx may set up so mild a reaction in the host that it attracts no notice In other instances an acute inflammatory process may progress to the stage of a purulent rhinitis

Meningococcemia presents the picture of acute sepsis with fever chills malaise and prostration Usually, the typical rash can be detected early in the disease This consists of dusky red spots or petechiae of varying sizes up to 15 mm in diameter which involve the skin and the mucous membranes of the body In severe cases the petechiae may assume a purpuric appearance as the result of hemorrhage These may reach a diameter of several centimeters and go on to actual necrosis In mild cases the spots disappear within a few days leaving small brownish areas in their wake histologic examination has revealed that these lesions are due to thrombo-embolic involvement of the capillaries Puncture of the lesion frequently demonstrates the presence of meningococci Fulminant infections often give rise to the Waterhouse Friderichsen syndrome characterized by circulatory collapse and shock Diffuse hemorrhage into and necrosis of the adrenal glands are occasionally found at autopsy, but these appear to be associated rather than essential pathologic lesions (Ferguson and Chapman 1948) In the absence of this complication recovery is the general rule if the disease is treated vigorously and promptly

Invasion of the central nervous system is marked by signs and symptoms of meningeal irritation and inflammation Severe headache pain in the posterior aspects of the neck on forward flexion nausea vomiting and often coma are prominent features Physical examination reveals muscular spasm stiff neck exaggerated reflexes and positive Kernig and Brudzinski signs Convulsions and bulging fontanelles are common findings in infants

The meningitis is characterized by an acute inflammatory reaction accompanied by thromboses of the smaller blood vessels and hemorrhage In the later stages marked thickening of the meningeal coverings is a prominent feature as the originally purulent exudate becomes organized Encephalitic involvement is characterized by focal areas of hemorrhage thrombosis and perivascular infiltration

IMMUNITY

The mechanism of recovery from clinically recognizable meningococcal infections has not been determined During the course of the clinical illness antibody formation may be demonstrated by a variety of technics including agglutination mouse protection and bactericidal tests among others The role that this antibody formed during the course of the

TABLE 2 DIFFERENTIAL CHARACTERISTICS OF *Neisseriae*

ORGANISM	APPEARANCE OF COLONY AFTER 24 HOURS INCUBATION	GROWTH ON PLAIN NUTRIENT AGAR	GROWTH AT 22 C	FERMENTATION: DEXTROSE	FERMENTATION: MALTOSE	FERMENTATION: SUCROSE	OTHER
N meningitidis	Round smooth glistening translucent colorless creamy consistency	—	—	+	+	—	4 distinct antigenic groups serologically
N gonorrhea	Similar to *N meningitidis* Smaller and more opalescent	—	—	+	—	—	
N catarrhalis	Smooth glistening translucent or firm somewhat opaque and adherent May be difficult to emulsify	+	+	—	—	—	Often agglutinates in normal horse serum or saline
N flavescens	Yellow pigmentation when first isolated otherwise similar to *N meningitidis*	+	±	—	—	—	Homogeneous distinct group serologically
N sicca	Small somewhat opaque wrinkled colonies quite brittle	+	+	+	+	+	Spontaneous agglutination in saline and normal horse serum

for 2 hours in a water bath before being examined A final confirmatory examination may be carried out after overnight incubation at 4° C

Growth characteristics fermentation reactions and serologic tests serve to differentiate other members of the genus *Neisseria* from the meningococci (Table 2) *Neisseria catarrhalis* and *N sicca* are characterized by their ability to grow at room temperature The latter forms rough dry appearing colonies and both species tend to agglutinate spontaneously in saline or normal horse serum Of the various pigmented species of Neisseria only *N flavescens* appears to be of any importance since it was isolated from a small epidemic of meningitis in Chicago by Branham (1930) The growth characteristics of the gonococcus closely resemble those of the meningococcus As regards biochemical reactions *N catarrhalis* and *N flavescens* fail to ferment dextrose maltose and sucrose and the gonococcus ferments only dextrose On the other hand *N sicca* ferments all 3 sugars Agglutination tests will usually demonstrate the antigenic specificity of these organisms Since all *Neisseria* possess the oxidase which attacks dimethyl or tetramethyl paraphenylene diamine hydrochloride this reaction is of little diagnostic value except in instances of cultures taken from the nasopharynx where it may be helpful in the recognition of organisms presumably falling into the genus *Neisseria*

TREATMENT

Serum therapy was introduced by Jochmann in 1906 and its use on a wide scale was stimulated by the report of Flexner in 1913 With the recognition of serologic groups among the meningococci came the development of polyvalent sera for the treatment of the disease with a concomitant improvement in results Although serum therapy is immunologically sound and the beneficial results obtained unquestioned it has found little use since 1940 because of the superior efficacy and ease of administration of chemotherapeutic agents such as the sulfonamides and penicillin

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Spinal fluid is collected by lumbar puncture in the 4th lumbar interspace The spinal fluid is permitted to drop directly onto the surface of plates of casein hydrolysate blood agar or chocolate agar medium It is well to inoculate a tube of broth in addition and most authorities recommend the incubation of a few ml of spinal fluid collected in a sterile test tube since on occasion positive results will be obtained in this material on subinoculation when the other cultures remain negative Finally approximately 5 ml is collected for physical examination including cell count and smear and in addition immunologic tests A marked polymorphonuclear pleocytosis is the usual finding The fluid is centrifuged and the sediment stained with methylene blue in order to reveal the typical extracellular or intracellular diplococci It is imperative that this examination be made promptly because of the tendency of these organisms to undergo autolysis In those instances where organisms are found in great numbers the addition of a small amount of specific antiserum may result in the demonstration of capsular swelling and the consequent serologic identification of the causative organism A small amount of the supernatant fluid may be transferred to a small precipitin tube in which it is layered gently over an approximately equal amount of monovalent antimeningococcal serum Frequently a positive precipitin reaction may be obtained in cases of severe infections in which the organism has been demonstrated in the sediment. Occasionally a positive reaction may be obtained even when no organisms are visible in the smear The reaction observed is usually specific for the various serologic groups of meningococci and thus affords a rapid method of diagnosis

Since meningococcal meningitis represents a purulent form of the disease the spinal fluid shows a predominance of polymorphonuclear leukocytes The finding of such a fluid in the course of an epidemic should be taken as presumptive evidence of infection due to the meningococcus until proved otherwise The general inflammatory nature of the meningeal reaction is reflected by the increase of spinal fluid protein and by the abnormal colloidal gold curve If organisms are present in significant numbers, the spinal fluid sugar level is reduced accordingly as in the case of other pyogenic forms of meningitis Nasopharyngeal cultures are of importance since in most cases of meningococcal meningitis and meningococcemia cultures taken from this area yield positive results In such instances the organisms isolated from the blood or the spinal fluid and from the nasopharynx are almost inevitably of the same group The culture is taken by means of a bent wire swab with cotton wool on the end This is inserted carefully behind the uvula and the soft palate so as to avoid contamination from other oral structures and is rubbed gently over the surface of the posterior nasopharynx. Then the swab is drawn over part of a suitable plate and the streaking is completed by means of a wire loop Prompt inoculation and incubation of these plates are of importance however if this is impossible the meningococci may be preserved by inserting the swab into a small amount of sterile horse blood contained in a sterile tube Under these conditions apparently there is no significant diminution in the number of viable organisms over a period of some hours If positive blood and spinal fluid usually yield the organism in pure culture frequently nasopharyngeal cultures give the same results Suspicious colonies and broth cultures may be subcultured for the identification of the specific organism by means of gram stain fermentation tests and serologic typing The morphologic appearance of the meningococcus on smear has been described These organisms usually ferment maltose and dextrose although the reaction may be delayed and somewhat erratic in the case of freshly isolated strains Sucrose is not fermented For serologic identification suspicious colonies are emulsified in small amounts of saline which usually contain 0 1 per cent potassium cyanide to inhibit the autolytic process The addition of group-specific antisera A B or C usually results in the specific identification of the organism by means of the agglutination test Strains isolated from the nasopharynx during interepidemic periods are frequently untypable Error due to nonspecific agglutination should be guarded against by the use of a normal control serum diluted 1 50 and of a saline suspension of the organism The tube agglutination test has been found to yield reliable results the suspension is shaken at room temperature and incubated at 37° C

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laboratory methods for the isolation and the identification of the organism have increased our knowledge of the epidemiology of meningococcal infections. Inapparent or subclinical cases far outnumber clinically recognizable cases. "Healthy" carriers are fairly common in both interepidemic and epidemic periods, yet even in the latter relatively few of the "carriers" develop clinically recognizable disease. A positive correlation between the carrier rate and the incidence of clinical cases has long been postulated, but careful analyses carried out in World War II (Pharr and Schoenbach 1944; Aycock and Mueller 1950) have shown that no such relationship exists.

The vast majority of epidemic outbreaks of meningococcal meningitis are due to Group A strains, although small outbreaks due to Group C strains have been reported. Sporadic cases of the clinical disease are usually due to Group B or C strains. Cases due to Group D strains are so rare that the data are inconclusive. These facts are reflected in the carrier rates. In interepidemic periods, Group B strains represent the predominating organism isolated from carriers; in actual fact, Group A strains are marked by their rarity. However, in epidemic periods, strains belonging to this latter group may furnish a significant proportion of the organisms isolated from apparently healthy individuals.

The clinical disease has long been associated with military camps and barracks, and, in these, recruits have appeared to suffer far more than seasoned troops. Immunologic inexperience and susceptibility to excessive fatigue consequent upon the "hardening" process of the raw recruit have been given as factors influencing this difference of incidence, but convincing evidence is lacking. No doubt overcrowding, a situation often found among military and civilian groups during wartime conditions, favors the spread of the organism from one individual to another (Hirsch 1886). However, even during the course of an epidemic in a closed group, it is difficult to find evidence of spread from one *clinical* case to another. The epidemiology of meningococcemia and meningococcal meningitis will not be clear until some understanding and recognition are obtained of those factors in the host which permit or favor the development of the clinically innocuous carrier state into a full-blown case of a dramatic and dangerous disease.

PREVENTION

The extreme degree of sensitivity of the meningococci to the action of the sulfonamide compounds has rendered chemoprophylaxis the most effective method of preventing clinical cases of meningococcal infection in the past. The efficacy of small doses of sulfadiazine in clearing the carrier state was demonstrated early in World War II, and the knowledge was quickly applied to the institution of chemoprophylactic measures on a large scale, with gratifying results. While small doses resulted in the prompt disappearance of meningococci from the nasopharynx, there was a general tendency for the organisms to reappear after an interval of a few days. Larger prophylactic doses resulted in the disappearance of the organisms for appreciably longer period of time. The results of continuous prophylaxis (1 Gm of sulfadiazine per day over a period of months) may be illustrated by the experience of United States naval recruits in World War II. Although the primary object of chemoprophylaxis was the reduction of infections due to beta hemolytic streptococci, the virtual elimination of meningococcal meningitis in the recruits during an epidemic period was the most impressive result.

In the light of these results it seems wise to give close contacts of frank cases of meningococcal disease brief courses of chemoprophylaxis with sulfisoxazole or other soluble sulfonamide compounds. This is particularly important if (1) the close contacts are children and (2) exposure occurs during an epidemic period. In the case of hypersensitivity to these compounds, penicillin provides an adequate substitute. Presumably the administration of a longer course of chemoprophylaxis to the members of a closed community would result in an appreciable period of freedom from these infections. Such a program should not be initiated without due consideration for the potential complications arising from the emergence of drug-resistant strains, not only of meningococci but of other bacterial pathogens as well in

the disease Sulfisoxazole (Gantrisin) has been the preferred drug because of its relatively low toxicity The equally effective sulfadiazine entails the risk of crystalluria particularly when administered parenterally although this hazard may be markedly reduced by alkalinization of the urine and maintenance of an adequate renal output The average uncomplicated case responds promptly to the administration of either of these sulfonamides when treatment is initiated early in the course of the disease If the patient is conscious oral administration of the drug will usually suffice although frequently a priming dose of parenterally administered drug is given at the onset of treatment in order to build up adequate levels in the blood as soon as possible In the unconscious or critically ill patient the drug must be administered parenterally Specific therapy must be maintained for several days after the patient s clinical course has returned to normal

Until recently primary sulfonamide resistant strains of meningococci were unknown and the emergence of drug resistant strains during therapy was not a problem In 1963 relatively high meningococcal carrier rates due to sulfonamide resistant strains of Group B were detected in 3 military installations in the United States (Millar 1963) This has led to a careful re-examination of the whole problem including the testing of many strains of the organism for sulfonamide sensitivity With one exception all strains of Group C have shown exquisite sensitivity to these drugs in the absence of recent outbreaks due to Group A organisms no new strains of this type have been available for testing Many but not all Group B strains have shown a slight but definite degree of resistance in the 3 instances described above resistance to sulfonamides was complete Further studies are required in order to determine if Group B meningococci are distinguished by their latent ability to develop resistance to the sulfonamide series of drugs The practical implication of these recent findings is that the critically ill patient or the one who fails to respond promptly and dramatically to sulfonamide therapy should be treated with a combination of agents Since excellent therapeutic results have been obtained with penicillin this is usually the drug of choice for combination with sulfisoxazole or sulfadiazine The broad spectrum antibiotics (notably chloramphenicol) are also effective Resistance to these agents or to penicillin has not been reported as yet.

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EPIDEMIOLOGY

Man represents the only recognized reservoir of the meningococcus The portal of entry and exit is the upper respiratory tract and the means of spread is essentially person to person via air borne droplets or by inanimate objects which are contaminated by the nasopharyngeal secretions of individuals carrying the organisms The extreme sensitivity of the organism to adverse physical conditions such as heat and low humidity means that intimate contact between two individuals is necessary for its spread

The clinical disease occurs both sporadically and in epidemics (Hirsch 1886) The latter show some evidence of cyclical occurrences (Gover and Jackson 1946 Hedrich 1952) with peaks of high prevalence occurring at 8 to 12 year intervals The incidence is usually highest in the late winter and early spring months summer and autumn show a significantly lower incidence of cases Young children are particularly at risk with some evidence suggesting a higher incidence in males than in females The case fatality rates are highest among the young and the aged

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The identification of the causative organism the discovery of the multiplicity of antigenic types and the development of effective

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19

The Gonococcus

INTRODUCTION

The gonococcus (*Neisseria gonorrhoeae*) is a gram negative diplococcus a species of the genus *Neisseria* to which belong also the meningococcus and several nonpathogenic inhabitants of mucous membrane including the anaerobic *Veillonella* (family Neisseriaceae)

The gonococcus is the cause of a number of contagious infections of human columnar and transitional epithelium Hence urethritis cervicitis salpingitis and other complications in adults vulvovaginitis in children and ophthalmia in the newborn and in adults are contagious inflammatory conditions all caused by the gonococcus The commonest is gonorrhea known in the vernacular as clap or strain

HISTORY

While most authors suggest that gonorrhea is a disease of great antiquity there is no conclusive proof to support this contention Ancient Egyptian prescriptions early Chinese and Japanese writings Biblical and Vedic references are suggestive but all of them are so vague that it is impossible to determine whether the writers had in mind a specific contagious urethritis or not The clearest descriptions of the progress do not suggest any inflammatory component or contagious character of the urethritides described Galen A D 130 first employed the term gonorrhea which may be translated as flow of seed As Vertue (1953) suggests Galen s own definition and description of gonorrhea is equivalent to what today is termed spermatorrhoea Support of this contention may be found in the contemporary writings of independent physicians such as Aretaeus the Cappadocian and Celsus of the first century A D From Hippocrates to Galen and still later the medical profession showed no general recognition of venereal disease When a contagious urethritis began to appear a place for it consistent with Galen had to be found Quite logically the term gonorrhea was chosen to carry the burden Gulielmus de Saliceto a 13th century physician of Placenza presumably dealt with this disease and was aware of its venereal origin

* The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of Health Education and Welfare or the U S Public Health Service

the course of prolonged chemoprophylaxis (Cheever 1945) If sulfonamide resistant strains of meningococci appear in significant numbers (Feldman 1963) this form of chemoprophylaxis will be sharply limited in value

As in the case of other air borne infections characterized by a high carrier rate attempts to prevent the spread of the causative organism by isolation and environmental control measures have met with little success No vaccine is available

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19

The Gonococcus

INTRODUCTION

The gonococcus (*Neisseria gonorrhoeae*) is a gram negative diplococcus a species of the genus *Neisseria* to which belong also the meningococcus and several nonpathogenic inhabitants of mucous membrane including the anaerobic *Veillonella* (family Neisseriaceae)

The gonococcus is the cause of a number of contagious infections of human columnar and transitional epithelium Hence urethritis cervicitis salpingitis and other complications in adults vulvovaginitis in children and ophthalmia in the newborn and in adults are contagious inflammatory conditions all caused by the gonococcus The commonest is gonorrhea known in the vernacular as clap or strain

HISTORY

While most authors suggest that gonorrhea is a disease of great antiquity there is no conclusive proof to support this contention Ancient Egyptian prescriptions early Chinese and Japanese writings Biblical and Vedic references are suggestive but all of them are so vague that it is impossible to determine whether the writers had in mind a specific contagious urethritis or not The clearest descriptions of the progress do not suggest any inflammatory component or contagious character of the urethritides described Galen A D 130 first employed the term gonorrhea which may be translated as flow of seed As Vertue (1953) suggests Galen s own definition and description of gonorrhea is equivalent to what today is termed spermatorrhoea Support of this contention may be found in the contemporary writings of independent physicians such as Aretaeus the Cappadocian and Celsus of the first century A D From Hippocrates to Galen and still later the medical profession showed no general recognition of venereal disease When a contagious urethritis began to appear a place for it consistent with Galen had to be found Quite logically the term gonorrhea was chosen to carry the burden Gulielmus de Saliceto a 13th century physician of Placenza presumably dealt with this disease and was aware of its venereal origin

* The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of Health Education and Welfare or the U S Public Health Service

He attributed the affliction to impurities retained under the male prepuce after exposure to an unclean female and was the first to suggest prophylaxis by washing In a manuscript of 1376 by John of Arderne (John Arden) who was surgeon to Richard II and Henry IV, is found one of the first recorded descriptions of contagious urethritis comparable with contemporary gonorrhea In the following 3 centuries it is obvious that the initiated were well aware that this entity was a venereal contagion However a division arose in medical thinking as to whether gonorrhea was a separate disease or a manifestation of syphilis The history of gonorrhea began merging with syphilis on the appearance of the latter as a recognized entity in Western Europe at the close of the 15th century As early as 1530 Paracelsus taught that gonorrhea was an initial symptom of syphilis Indeed in those times the observed relationship was probably correct more often than not The concept that gonorrhea was a manifestation of syphilis reached its pinnacle of acceptance after the classic error of John Hunter who in 1767 misinterpreted the syphilis infection which resulted from self inoculation with pus from the urethra of a patient supposedly infected with gonorrhea Hunter s work so dominated medical thought that in spite of the excellent work of Hill in 1790 and Benjamin Bell in 1792 clearly differentiating gonorrhea from syphilis it remained for Phillippe Ricord in work extending from 1831 to 1860 to delineate the 2 diseases clearly and finally Noeggerath in 1872 published one of the first works discussing the prevalence of gonorrhea especially in women its resistance to therapy its highly infectious nature the insidious tendency to remain latent for prolonged periods and the serious nature of complications and sequelae In 1879 Neisser identified the causative organism of this disease which he called the gonococcus Subsequent to its discovery the gonococcus was first cultivated by Leistikow and Loeffler in 1882 but was more satisfactorily grown and studied by Bumm in 1885 The identification of the organism was simplified generally but not specifically by the introduction of the gram stain by Hans Gram in 1884 Finger Ghon and Schlagenhaufer in 1894 published a description of the histopathology of the disease based on the study of postmortem material obtained from patients artificially infected in the terminal stage of other diseases Muller and Oppenheim in 1906 applied the principles of complement fixation successfully to the diagnosis of gonorrhea Diagnostic technics in general improved little beyond this point until the recent observation that the fluorescent antibody technic was adaptable to the identification of *N gonorrhoeae* Treatment was advanced from the era of sandalwood oil by the introduction of potassium permanganate solutions for urethral irrigations by Janet in 1892 For over 4 decades only minor modifications of this basic technic were introduced in the treatment of the disease With the advent of the sulfonamides specific therapy seemed to be at hand but within a few years the optimism concerning these drugs was blasted by the ability of the gonococcus to become resistant to these drugs However the very striking susceptibility of *N gonorrhoeae* to penicillin was demonstrated in 1943 Confidence in the penicillin treatment of gonorrhea led to complacency not only concerning the ease of treatment of this disease but also in relation to curability and the complications which were less and less frequently seen in hospitals and clinical practice With the advent of penicillin many assumed that gonorrhea would be brought under control readily That this has not occurred can be demonstrated by reference to our national morbidity statistics The fallacy of the supposition that any disease can be controlled by treatment alone without due consideration of other aspects of its epidemiology is beautifully borne out by our experience with gonorrhea to date Until recently, about all that had been gained in the control of this disease during the little more than 85 years since the discovery of the gonococcus were some minor improvements in diagnosis a highly effective therapeutic agent which may have been squandered by our ignorance and an almost total prevention of serious complications of the disease in males Gonorrhea remains the largest and most challenging venereal disease problem in the western world

MORPHOLOGY

The gonococcus appears in the exudate of *acute gonorrhea* as a diplococcus with contiguous sides flattened or slightly concave resembling a pair of kidney beans and measuring from 0 6 to 1 0 micron in diameter In these exudates many polymorphonuclear leukocytes contain no ingested organisms while others display from a few to large numbers The gonococcus does not possess spores true capsules or flagella It is stained readily by aniline dyes and is gram negative Ordinary optical methods do not reveal any intracellular differentiation

Cells growing in laboratory media differ somewhat in appearance from those seen in pathologic material Cultures consist predominantly of diplococci but may also contain single cocci and clumps Older cultures frequently exhibit irregular staining giant forms Cellular morphology is altered progressively by contact with penicillin both in vitro and in vivo Smears of exudate taken during the first 4 or 5 hours of penicillin therapy show gonococcal forms that progressively enlarge and decrease in stainability until none can be detected

CULTIVATION AND CHARACTERISTICS

Primary cultivation of the gonococcus on laboratory media is difficult not only because the organism is fastidious in its growth requirements but also because it is exceedingly susceptible to the toxic effect of a variety of substances commonly present in ordinary media The organism grows best under aerobic conditions at pH 7 2 to 7 6 at a temperature of 35 to 36 C Some strains do not grow satisfactorily at 37 5 C and in general growth stops below 30 C or above 38 5 C Most strains require an atmosphere containing from 2 to 10 per cent CO to initiate development Although gonococci grow well on the moist surface of solid media containing 1 0 to 1 5 per cent agar excessive moisture as produced by syneresis of the agar is undesirable especially for primary isolation because it favors other bacteria especially spreaders which readily overgrow the more slowly growing gonococcus Satisfactory growth is obtained on agar media consisting of meat infusion peptones glucose buffered with phosphate and enriched with plasma and hemoglobin or whole blood Increased yields can result from the addition to the media of yeast or liver concentrate which supply glutamine and cocarboxylase shown by Lankford *et al* (1943 1946) to be essential for 10 to 15 per cent of gonococcus strains Kellogg *et al* (1963) have shown that hemoglobin solution or blood may be replaced by adding ferric nitrate to Lankford s supplement of glutathione cocarboxylase and glucose As mentioned earlier many components of media exert an inhibiting effect on the growth of gonococci Thus certain amino acids occur in peptones in concentration sufficient to be somewhat toxic but this effect can be reduced markedly or abolished by heating the medium after addition of blood (chocolate agar) Ley and Mueller (1946) have shown that agar containing excessive amounts of fatty acids also can exert an inhibiting effect on the growth of certain strains of gonococci and that the effect can be counteracted by charcoal or starch Advantage has been taken of all these facts in certain commercial media which are convenient for the isolation of gonococci from pathologic material The bacterial flora of urethral and cervical secretions contain organisms that grow very much more rapidly on artificial media than does the gonococcus Such overgrowth prevents the detection of gonococcus colonies Attempts to prepare a selective medium by the use of Nile Blue A or crystal violet were not very successful because these dyes were toxic to some gonococcal strains Recently an effective selective medium for the cultivation of *N gonorrhoeae* or *N meningitidis* has been developed by Thayer and Martin (1963) It has selective properties that permit cultivation of pathogenic *Neisseria* while greatly inhibiting or completely suppressing saprophytic species such as *N sicca N catarrhalis N flava* and others Overgrowth of gonococcal colonies by bacterial contaminants encountered in cervical vaginal and even rectal specimens is almost totally prevented and the diagnostically confusing *Mima polymorpha* var *oxi*

dans a microorganism sometimes confused with gonococci fails to grow Thus, false positive cultural diagnosis of gonorrhea due to oxidase positive *Mima* and saprophytic *Neisseria* is almost totally prevented Pre sumptive cultural testing (oxidase positive colonies of gram negative diplococci uncon firmed by sugar fermentation reaction) there fore becomes a more acceptable diagnostic procedure when definitive measures cannot be taken The Thayer Martin (TM) selec tive medium is of particular value for diag nosing the meningococcal carrier state Bac teria of the pharyngeal region including saprophytic *N catarrhalis N sicca* and others are almost totally inhibited allowing meningococci to grow and be picked from primary isolation plates for typing with group sera without the necessity of purification pro cedures The selective properties of the me dium are due to the action of polymyxin B (25 units/ml) against the gram negative bacterial flora and to the inhibition of the gram positive bacterial flora by ristocetin (10 mcg /ml) In the concentrations used gonococcal growth is slightly inhibited during the first 24 hours of incubation but not after 48 hours After 48 hours incubation the primary gonococcus colony appears trans lucent raised finely granular and slightly convex with lobate margins It is usually mucoid and varies in size from punctiform to 5 mm in diameter depending on the me dium and the crowding of the plate Although ordinary agar cultures die within 3 to 4 days unless transferred agar-slant cultures remain viable at 35° C for a considerable period of time if covered with sterile paraffin oil Cul- tures may also be kept for years if quick frozen and stored at −70° C

Glucose is the only sugar fermented by the gonococcus with the production of acid but no gas Other members of the genus *Neisseria* are encountered occasionally in specimens from the genital tract Fermentation reactions differentiate these species from the gonococ cus Some gonococcal strains fail to grow on media lacking adequate nutrients and a few isolates fail to ferment glucose initially (White *et al* 1964) Thus some so-called infections thought to be due to *N catarrhalis* may have been caused by non glucose fermenting gono- cocci Differentiation of such strains should include immunofluorescent studies (Gono coccus 1962)

The fact that the gonococcus produces an oxidase has been utilized for the recogni tion of gonococcal colonies on agar medium When 1 per cent aqueous solution of di methyl p phenylenediamine monohydrochlo ride is added to an agar growth the colonies turn pink then purple The reagent kills the organisms within a few minutes but does not modify their morphology or staining by Gram s or the fluorescent antibody method The oxidase reaction is often negative in media containing 1 per cent glucose due to inhibition of the enzyme by acid neutraliza tion with sodium bicarbonate results in a positive oxidase test (Bucca Thayer and Schubert, 1947) All members of the genus *Neisseria* give positive reactions, so do *Mima polymorpha* var *oxidans* some *Pseudo monas* and *Aeromonas* Variable results are given by *Bordetella Haemophilus* and some *Pasteurella* and yeasts (Steel 1961) These microorganisms may confuse the laboratory identification if adequate measures are not taken Nevertheless a positive oxidase re action coupled with typical colonial char acteristics and the presence of diplococci resembling the gonococcus constitutes pre sumptive cultural evidence which should be confirmed by sugar fermentation and/or fluorescent antibody procedures

As mentioned under ordinary conditions the gonococcus dies rapidly in agar cultures It is killed quickly by drying sunlight and ultraviolet light Moist heat at 55° C kills it in a few minutes Phenol bichloride of mercury and silver compounds are very effec tive disinfectants The gonococcus is more soluble than the meningococcus in dilute NaOH (within 1 minute) whereas other *Neisseria* are less soluble in this reagent Al though the gonococcus is susceptible to sul fonamides the range may vary from com plete resistance of certain strains which can synthesize para aminobenzoic acid to sus ceptibility to drug concentration readily ob tained in the blood Gonococcal strains for merly highly resistant to sulfonamide have again become sensitive during the era of penicillin therapy (Love and Finland, 1955

Thayer Field and Garson 1959) Resistance to sulfonamides and streptomycin is rapidly acquired in vitro

For almost a decade it appeared that the gonococcus would not become resistant to penicillin as it had to sulfonamides From 1945 to 1954 susceptibility studies in this country and abroad showed that of 771 cultures all but 5 strains were sensitive to 0 05 u /ml or less (Guthe 1961) In trying to explain the high failure rate of females treated with 600 000 units of procaine penicillin Thayer *et al* (1957a) found that 22 per cent of the patients cultures required 0 1 to 0 2 u /ml to inhibit in vitro growth Surveillance studies of treatment failure cases since that time have shown that the range of penicillin resistance is slowly expanding To day some isolates require over 1 0 u /ml for in vitro inhibition (Thayer 1963) Of the semisynthetic penicillins tested only ampicillin is as potent as benzyl penicillin G for sensitive and relatively resistant gonococci (Thayer and Axnick 1963b) In tissue culture intracellular gonococci may be protected against many times the minimal inhibitory concentration of penicillin chloramphenicol erythromycin novobiocin and the tetracyclines (Thayer *et al* 1957b c)

The gonococcus causes infection only in man numerous attempts to reproduce the disease in animals have failed However under certain conditions growth of minimal numbers of gonococci has occurred in embryonated eggs in the anterior chamber of the eye of the rabbit and in the mouse by intraperitoneal injection of gonococci suspended in mucin (Hill 1944) Kellogg and co workers (1963) have found that gonococci can be maintained virulent in culture Four morphologically distinct colonial types have been observed Type 1 is of most interest since it is found predominantly in the purulent exudate from acute gonorrhea of the male Other colonial types appear rapidly on subculturing the primary isolate probably due to genetic instability of the type 1 cells in vitro After 69 passages on suitable culture media type 4 cells typical of so-called laboratory strains were no longer virulent for male volunteers whereas type 1 cells readily produced gonococcal urethritis In the past studies in serodiagnosis and immunity have been performed with avirulent cultures

The toxicity of the gonococcus appears to be due entirely to a lipopolysaccharide endotoxin (Tauber and Garson 1959) The injection of heat killed gonococci results in toxemia and death similar to that produced by living cocci So far other chemical components separated from the gonococcus have not proved to be useful in the development of diagnostic tests Failure to detect significant levels of antibodies consistently by agglutination precipitation bactericidal complement fixation and allergy tests has been related to the fact that the infection stimulates little antibody formation because of its local character Genetic instability of the virulent culture also may have resulted in the preparation of nonspecific antigens (Reising and Kellogg 1963) However in controlled experiments volunteers displaying a positive gonococcus complement fixation test prior to inoculation did not develop disease with the same frequency as those recorded as having a negative test (Mahoney 1946)

CLINICAL COURSE

The usual incubation period of the natural infection is from 2 to 8 days and varies from 1 to 31 days with a mean of from 3 to 5 days in the experimental disease (Mahoney *et al* 1946) These experimental observations closely parallel clinical observations (Garson 1953 Haro and Patiala 1957)

The typical onset is sudden Usual symptoms consist of frequent urgent and painful urination and a profuse mucopurulent discharge The gonococcus is unable to penetrate stratified squamous epithelium however in the male the urethra with its stratified columnar epithelium is favorable for its penetration (Harkness 1948) Penetration takes place through the intercellular spaces the organism being observed to reach the subepithelial connective tissue on the 3rd or the 4th day Polymorphonuclear leukocytes lymphocytes plasma and mast cells soon appear beneath the columnar epithelium being particularly numerous in the region of Littre s glands and ducts and the lacunae of Mor

gagni Large numbers of leukocytes carrying gonococci find their way from the acutely inflamed area into the lumen of the urethra and form with serum the profuse yellow discharge characteristic of the disease The ducts of Littre s glands may become obstructed by leukocytes and desquamated epithelial cells resulting in the formation of retention cysts *or abscesses* Spread of the disease takes place by continuity from the subepithelial connective tissue and also directly into lymphatic vessels resulting in prostatitis and epididymitis Extension of pathology to subepithelial tissues and subsequent healing allows for con*tracture* which in annular structures may cause narrowing and stricture During heal*ing* stratified squamous epithelium appears over the granulating surface of denuded areas and may compress and destroy any underlying columnar epithelium

In the female the cervical glands Skene s glands and Bartholin s glands are usual sites of primary infection The rectum is frequently the site of secondary infection and occasionally the primary site Histologic pathology is similar to that described for the male urethra Involvement of cervical glands as for Littre s glands usually is confined to the ducts giving rise to a mucopurulent discharge of a degree ranging from mild to very severe

Progression to the fallopian tubes results in salpingitis usually bilateral There is a tendency for the tubes to close and form pyosalpinx and pelvic inflammatory disease Residual pathology in the tubal structures may require surgical correction after the inflammatory process has been brought under control In the glands of Bartholin unilateral inflammation is confined to the ducts and the periglandular tissue

The incidence of proctitis determined with the TM selective medium is found in 40 per cent of urogenital female gonorrhea An additional 5 per cent occurs at the rectal site only Among patients with proctitis gonococci are also found at the perianal site in 50 per cent of the cases (Schroeter and Yobs 1964)

N gonorrhoeae can be responsible for ophthalmia neonatorum an inflammation of the eye of the newborn resulting primarily from infection during passage through the birth canal The condition, which appears several days after birth is always serious is frequently destructive *to the ocular struc*tures and before the use of silver nitrate prophylaxis was credited *with being respon*sible for 12 per cent of all blindness Today the recorded incid*ence is less than 0 3* per cent where standard technics of prophylaxis are used That silver nitrate prophylaxis does not entirely prevent gonococcal ophthalmia was found by Pearson (1957) *who observed* 40 cases in 67 200 live births during a period of 10 years The ease with which gonococcal ophthalmia responds to antibiotic treatment has led to a relaxation of prophylactic measures in areas where postnatal supervision is satisfactory The risk of chemical conjunctivitis from silver nitrate may be avoided by antibiotic prophylaxis (penicillin erythromycin oxytetracycline bacitracin ointment and sulfathiozole) However such measures have not proved to be entirely satisfactory and in view of the increased incidence of gonorrhea there has been a return to silver nitrate prophylaxis

Gonococcal vulvovaginitis is an inflammation of the urogenital tract of prepubescent females and must be differentiated by bacteriologic means from that due to a variety of other infectious agents The disease is transmitted by intimate direct contact with infected adults and infrequently by contact with contaminated moist articles Cohn *et al* (1940) believe that epidemics rarely occur

DIAGNOSIS

Gram negative intracellular diplococci in the stained exudate from a suspected gonococcic infection strongly suggests gonorrhea The intracellular position of the gonococcus is a common finding in acute gonorrhea but in very early or chronic infection the organisms may be found only extracellularly frequently as a single coccus In men a diagnosis based on the characteristic clinical symptoms usually can be confirmed by the finding of intracellular gram negative diplococci in pus cells in the urethral discharge Preferably such presumptive evidence of *N gonorrhoeae* should be confirmed by culture methods including identification by sugar fermentation

An outstanding diagnostic advance is the

immunofluorescent method for the detection and the identification of gonococci in exudate The procedure developed by Deacon *et al* (1959 1961) employs an immediate direct fluorescent antibody (FA) method and a delayed FA technic In the former complete identification as *N gonorrhoeae* may be obtained within 1 hour The delayed FA method which is more effective than the immediate direct method for diagnosing the asymptomatic female (Brown *et al* 1962) requires a 16 to 20 hour preliminary cultivation of exudate to accumulate gonococci in sufficient numbers for detection by fluorescent microscopy The chief advantages of the delayed FA procedure over the conventional clinical culture method are the time required to make a definitive diagnosis—16 to 20 hours against 2 to 10 days respectively—and the greater sensitivity of the technic in most laboratories In a field evaluation of the FA method by public health and university laboratories (Price 1964) over 85 000 specimens from urethral cervical and vaginal sites of 9 483 females were examined Using immediate direct and delayed FA methods and the classic culture method gonococci were detected by culture in 22 per cent by delayed FA in 37 per cent and by direct FA in 15 per cent When female contacts of persons having gonorrhea were studied the culture was positive in 59 per cent immediate direct FA 43 per cent and delayed FA in 79 per cent of 1 782 patients Some workers (Moore *et al* 1963) feel that the FA procedure occasionally gives a false positive result Peacock (1961) has greatly reduced the likelihood of false positive staining of staphylococci by applying the one step inhibition technic for blocking antistaphylococcal antibodies present in normal rabbit serum Peacock (1964) has also controlled background and polymorphonuclear cell staining with Flazo Orange With the use of this dye or Evans Blue (White and Kellogg 1964) counterstained direct FA smears prepared on a small defined area of the slide with a bacteriologic loopful of specimen increases the sensitivity of the procedure

Trauma or the introduction of mechanical and chemical irritants into the urethra may give rise to an inflammatory process which has some of the characteristics of gonorrhea but can be differentiated by negative bacteriologic findings Similarly a condition designated as nongonococcal urethritis or nonspecific urethritis is encountered Instead of a burning sensation during urination as with gonorrhea an itching sensation of the urethral canal and pain referred to the glands occurs independent of urination The discharge is thinner gray to white in color and usually less profuse than gonorrhea The presence of *N gonorrhoeae* or *Trichomonas vaginalis* must be ruled out

Mistakes in the microscopic diagnosis of gonorrhea may be made when gram positive cocci are overly destained or gram negative diplo bacilli are wrongly identified as gonococci Also the morphology of the *Mima Herella* group of organisms is quite similar to gonococci especially in exudates and from growth on enriched media Differentiation is easily made on meat extract agar unenriched broth or eosin methylene blue agar media on which *Mima* and *Herella* assume their rodlike form and on which the gonococcus does not grow Where gonococcal FA procedures are available differentiation is rapidly made Other than a single outbreak of a gonorrhealike syndrome caused by *Mimeae* in Italy (Svihus *et al* 1961) no microbial agent other than *N gonorrhoeae* has the capacity to produce acute infectious urethritis consistently in the human

Gonorrhea of the female may be signless and symptomless Thus laboratory diagnosis is more difficult than in men Stained smears are of value in early infections when typical intracellular organisms may be found in material from the urethra or the cervix As the age of the infection advances the value of the smear decreases while that of the culture and the delayed FA methods increases Fortunately the use of immunofluorescent methods and selective culture media has improved diagnosis but these procedures still fail to diagnose 20 to 40 per cent of female contacts of persons having gonorrhea (Price 1964) It should be pointed out in this connection that in chronic untreated gonorrhea culture findings may reverse spontaneously from positive to negative without any accompanying change in the clinical status of the infection Furthermore sporadic positive cultures may appear after a relatively

long series of negative cultures (Mahoney *et al* 1942)

Serodiagnosis using present methods of *complement fixation (gonoreaction) are not* sufficiently reliable to identify active disease in males or asymptomatic females (Van Slyke *et al* 1942 Nøgaard 1956 Brown, 1963) *Such a tool is urgently needed for the epi*demiologic control of gonorrhea

Schubert *et al* (1947) showed that gonorrheal exudate inoculated directly on the agar surface in the clinic and incubated shortly thereafter gave the greatest yield of positive cultures When this is not possible the swab containing the exudate should be placed in a small amount of broth and taken to the laboratory Difficulties inherent in the isolation of the gonococcus are increased when the clinician fails to exercise care in securing suitable exudates for cultivation The demonstration of gonococci in cervical exudate is said to be most favorable during the post menstrual phase However other workers prefer the premenstrual phase and some even choose the menstrual period Putkonen (1950) studied this problem in 343 patients hospitalized for gonorrheal cervicitis in whom *menstruation was known in relation to 1 119* examinations He found no significant variation of positive cultures in relation to the day of the menstrual cycle

The transportation of suspected secretions from the patient to a distant laboratory for cultural diagnosis has not been very successful An ideal transport medium must preserve *the viability of the gonococcus and at the* same time prevent overgrowth of contaminating bacteria Stuart (1954) uses a buffered nonnutrient agar medium to which thioglycollate is added In theory viable gonococci are preserved and contaminants fail to grow for lack of nutrients Specimens of blood synovial fluid and spinal fluid are cultivated best in shallow layers of ascitic fluid broth in an atmosphere containing from 2 to 10 per cent CO after incubation for 2 to 7 days the organisms grow as a mucoid sediment on the bottom of the flask Urine preferably the first morning specimen should be centrifuged and the sediment inoculated on agar medium Prostatic fluid may be inoculated directly on agar or centrifuged in a small amount of urine the sediment being inoculated on agar For more detailed information concerning the collection of specimens and the culture method see *Gonococcus—Procedures for Isolation and Identification* (1962)

In general, because of the characteristic clinical signs and symptoms, presumptive cultural evidence of gonococcal infection (unconfirmed by sugar fermentation) is more acceptable for diagnosis of gonorrhea in the male than in the female *In the female in* whom clinical evidence is of little value a negative smear report coupled with presumptive cultural evidence has led to as high as *16* per cent false positive diagnoses (Thayer *1958*) *Such* results are unlikely to occur, as already mentioned, if the TM selective medium is used (see Cultivation)

TREATMENT

The advent of chemotherapy in 1935 rapidly reduced such practices as the local application of antiseptic and astringent solutions by means of injection irrigations and instillations and of attempts to alkalinize the urine All of these methods are of question*able efficacy and may have added to the com*plications of gonorrhea Sera vaccines and culture filtrates have also proved to be of very limited if any value Artificial hyperpyrexia *based on the marked heat lability of the* gonococcus is no longer practiced When the sulfonamide compounds and subsequently penicillin became available the therapy of *gonorrhea passed from the province of urol*ogy and to a lesser extent, gynecology to that of chemotherapy largely in the hands of the general practitioner

Although the past recommended practice of employing 600 000 units of a repository penicillin in a single intramuscular injection for the routine treatment of uncomplicated gonorrhea has given excellent results in males Preston and Dunsworth (1957), Hookings and Graves (1956 57) and others have demonstrated at least a 13 to 15 per cent failure rate in females in clinic populations In general it has been alleged that as much as 2 to 5 per cent of males and 10 to 20 per cent of females failed to be cured by the single intramuscular injection of 600 000 units of *repository penicillin. The reason for such*

treatment failures in the face of the once rather remarkable susceptibility of the gonococcus to penicillin has been explored by Thayer *et al* (1957a 1962) Other than failures related to (1) misdiagnosis including nongonococcal urethritis other causes include (2) reinfection on return to the original infecting sexual environment (3) the use of drugs in inadequate doses or of drugs rendered useless by prior mishandling or deterioration (4) factors such as the effect of penicillinase producing staphylococci on male urethritis (Kjellander and Finland 1963) and the persistence of female proctitis (Bang 1954) and (5) the relative resistance of certain strains of *N gonorrhoeae* to penicillin (Thayer *et al* 1961)

The increasing incidence of treatment failure has led to modification of the recommended drug therapy regimen In place of the one shot treatment the following procedures are now recommended

Uncomplicated gonorrhea in men aqueous procaine penicillin G procaine penicillin G in oil with 2 per cent aluminum monostearate (PAM) 2 400 000 units in 1 intramuscular injection

Uncomplicated gonorrhea in women aqueous procaine penicillin G or PAM 4 800 000 units intramuscularly in 2 injection sites at one visit In view of the increased resistance of gonococcal strains to penicillin long acting penicillins which produce low blood concentrations such as benzathine penicillin G are no longer indicated in the management of most gonorrheal infections

Gonorrhea with severe complications generally should be treated with aqueous crystalline penicillin G 4 800 000 to 10 000 000 units per day intramuscularly given at 2 to 4 hour intervals or equivalent amounts of a penicillin producing a high blood level until signs and symptoms have subsided and cultures if obtainable are negative The management of most complications is more appropriately handled in the hospital where resources of consultation and special laboratory procedures are more readily and rapidly available

Retreatment usually is indicated if on the exclusion of the likelihood of reinfection the discharge persists for 3 days or more after initial treatment and smear culture or FA diagnosis is still positive In general the retreatment dose consists of a doubling of the original schedule at a single session or equally divided injections of the increased dose over a period of 3 to 5 days until the total dosage is achieved and signs and symptoms have subsided

The need for local therapy prostatic massage or artificial hyperpyrexia is seldom if ever encountered So-called prophylactic or epidemiologic treatment should be given to all sexual contacts of a positively diagnosed case of gonorrhea The recommended treatment for such contacts is as given for uncomplicated gonorrhea above

While there is no evidence of absolute or rapidly increasing resistance of *N gonorrhoeae* to penicillin studies of gonococcal strains in this country since 1955 show a relentless expansion of the susceptibility range varying today from 0 005 to 1 20 units per ml (Thayer and Moore 1964) The relatively resistant strains of the organism in cases of presumed treatment failure on routine dosage schedules usually will respond satisfactorily to increased dosage

Approximately 15 commercially available antibiotics are as effective as penicillin G at the 1 2 mega unit dose Indeed a single (1 5 Gm) oral dose of phosphate potentiated tetracycline was observed to have a failure rate in acute gonorrhea of males of 5 7 per cent as compared to 8 4 per cent with 1 2 mega units of aqueous procaine penicillin G given by intramuscular injection (Tiedemann 1962) Among the newer injectable antibiotics actinospectacin in a single intramuscular dose (1 5 to 2 0 Gm) has been observed to fail in between 4 3 and 7 9 per cent of cases however resistance to this drug has been reported (Laird 1962 Willcox 1962) Few of the newer antibiotics are serious competitors of penicillin in the treatment of gonorrhea when one considers such factors as mode of administration dose toxicity side-effects and cost They should be considered only in the treatment of patients known to be sensitive to penicillin

Brown (1961b) in a re-evaluation of reactions to penicillin studied 35 494 patients treated in venereal disease clinics and found that an average of 9 71 patients per 1 000

long series of negative cultures (Mahoney *et al* 1942)

Serodiagnosis using present methods of complement fixation (gonoreaction) are not sufficiently reliable to identify active disease in males or asymptomatic females (Van Slyke *et al* 1942 Nøgaard 1956 Brown 1963) Such a tool is urgently needed for the epidemiologic control of gonorrhea

Schubert *et al* (1947) showed that gonorrheal exudate inoculated directly on the agar surface in the clinic and incubated shortly thereafter gave the greatest yield of positive cultures When this is not possible the swab containing the exudate should be placed in a small amount of broth and taken to the laboratory Difficulties inherent in the isolation of the gonococcus are increased when the clinician fails to exercise care in securing suitable exudates for cultivation The demonstration of gonococci in cervical exudate is said to be most favorable during the post menstrual phase However other workers prefer the premenstrual phase, and some even choose the menstrual period Putkonen (1950) studied this problem in 343 patients hospitalized for gonorrheal cervicitis in whom menstruation was known in relation to 1,119 examinations He found no significant variation of positive cultures in relation to the day of the menstrual cycle

The transportation of suspected secretions from the patient to a distant laboratory for cultural diagnosis has not been very successful An ideal transport medium must preserve the viability of the gonococcus and at the same time prevent overgrowth of contaminating bacteria Stuart (1954) uses a buffered nonnutrient agar medium to which thioglycollate is added In theory viable gonococci are preserved and contaminants fail to grow for lack of nutrients Specimens of blood synovial fluid and spinal fluid are cultivated best in shallow layers of ascitic fluid broth in an atmosphere containing from 2 to 10 per cent CO_2 after incubation for 2 to 7 days the organisms grow as a mucoid sediment on the bottom of the flask Urine preferably the first morning specimen should be centrifuged and the sediment inoculated on agar medium Prostatic fluid may be inoculated directly on agar or centrifuged in a small amount of urine the sediment being inoculated on agar For more detailed information concerning the collection of specimens and the culture method see Gonococcus—Procedures for Isolation and Identification (1962)

In general because of the characteristic clinical signs and symptoms presumptive cultural evidence of gonococcal infection (unconfirmed by sugar fermentation) is more acceptable for diagnosis of gonorrhea in the male than in the female In the female in whom clinical evidence is of little value a negative smear report coupled with presumptive cultural evidence has led to as high as 16 per cent false positive diagnoses (Thayer 1958) Such results are unlikely to occur as already mentioned if the TM selective medium is used (see Cultivation)

TREATMENT

The advent of chemotherapy in 1935 rapidly reduced such practices as the local application of antiseptic and astringent solutions by means of injection irrigations and instillations and of attempts to alkalinize the urine All of these methods are of questionable efficacy and may have added to the complications of gonorrhea Sera, vaccines and culture filtrates have also proved to be of very limited if any value Artificial hyperpyrexia based on the marked heat lability of the gonococcus is no longer practiced When the sulfonamide compounds and subsequently penicillin became available the therapy of gonorrhea passed from the province of urology and to a lesser extent gynecology to that of chemotherapy largely in the hands of the general practitioner

Although the past recommended practice of employing 600 000 units of a repository penicillin in a single intramuscular injection for the routine treatment of uncomplicated gonorrhea has given excellent results in males Preston and Dunsworth (1957) Hookings and Graves (1956 57) and others have demonstrated at least a 13 to 15 per cent failure rate in females in clinic populations In general it has been alleged that as much as 2 to 5 per cent of males and 10 to 20 per cent of females failed to be cured by the single intramuscular injection of 600 000 units of repository penicillin The reason for such

available on incidence and prevalence nonetheless there remain tantalizing unknowns and inconsistencies The long known relationship of the social and behavioral patterns of promiscuity sexual freedom and irresponsibility to the continued presence of this disease is well established Gonococcal infection is maintained by the continued presence of the venereal form which in turn is maintained by indiscriminate sexual behavior

The distribution and the occurrence of gonorrhea is world wide Available data would suggest that it is more common among persons of lower socio-economic status but it is not restricted to this group It affects both sexes and practically all ages but particularly the age groups of greatest sexual activity In the United States the highest incidence is found in the age group 20 to 24 years Many states indicate that 25 per cent of the gonorrhea reported occurs among teenagers Studies of the U S Public Health Service have indicated that 50 per cent and more of the individuals involved in epidemics of gonorrhea are in the teenage group It has been estimated that in the United States a teenager is infected with venereal disease every 11 minutes The source of infection is exudate from mucous membranes of infected persons and man is the only known reservoir Asymptomatic carriers have been reported recently from England and the United States Although the number of such individuals is relatively small they are nevertheless important for the control of the disease The mode of transmission is almost wholly by sexual intercourse with the exception of the ophthalmic infection acquired by the newborn during passage through an infected birth canal Intimate contact on the proper type of mucous membrane is absolutely essential for the transmission of this disease and the role of intermediary objects in transmission seems to be inconsequential As indicated elsewhere the incubation period has wide variation while the period of communicability may be from months to years unless interrupted by adequate therapy which ends communicability within hours or days Susceptibility is general and instances of racial immunity have not been recognized The popular belief that an infection contracted from a member of a certain race or in a certain geographic area is more severe or more resistant to treatment than others has no factual basis Spontaneous recovery of the anterior urethritis in men is usual eventually in the absence of excessive sexual activity or reinfection Ricord s statement over a century ago is still applicable to recovery in women We know when gonorrhea begins but God alone knows when it will end Acquired immunity has not been demonstrated and one attack does not protect against subsequent infection

Males vary greatly in their resistance to experimental infection Mahoney *et al* (1946) inoculated a large number of male volunteers by instillation of massive doses of cultures into the urethral canal In a total of 245 experimental exposures typical clinical disease with confirmatory laboratory findings was produced in only 83 or 33 8 per cent In the remainder a train of irritative symptoms of varying severity that persisted for periods ranging from several hours to 2 days was followed by a return to normal More recently Kellogg *et al* (1963) and Thayer (1964b) have been able to infect male volunteers in 95 per cent of the trials by using gonococcal cultures of type 1 clones The relatively low incidence of the infection among prostitutes may possibly be the expression of such natural resistance although it is known that the well initiated among this group practice a wide variety of empiric prophylactic technics in the process of scrubbing the decks aimed at professional self preservation

In culture studies carried out over several years Van Slyke *et al* (1942) found approximately 20 per cent of incarcerated prostitutes and other sex offenders to be infected A similar percentage has been found among female jail inmates studied by the delayed FA test (Harris *et al* 1962) National morbidity statistics in the past two decades indicate a decline and then a rise in reported cases of gonorrhea From 1947 to 1956 there was a 40 per cent decrease in reported cases and a 47 per cent decrease in the rate Any optimism concerning this favorable trend in morbidity should have been tempered by the fact that during this period simplified antibiotic treatment was developed and more and more cases were seen by private physicians who failed to report

showed penicillin sensitivity, ranging from mild signs to severe anaphylactic reactions The prospect of having to handle the sudden emergency of penicillin anaphylaxis makes it essential that no physician or clinic under take penicillin treatment without the proper equipment drugs and professional com petence

Cure in men implies complete freedom from clinical evidence of the disease how ever an asymptomatic persistent infection can develop (Pariser 1964) Examination for evidence of residual gonococcic infection may be done about 7 days after the comple tion of therapy The test of cure should con sist of culture and FA procedures The ma terial for culture should be obtained from the urethra with a platinum loop and from sedi mented urine Expression of the urethral glands by gentle massage of the canal and prostatic massage has been employed In strumentation as an aid in determining the presence of gonococci is not necessary and is not advised In general a test of cure is considered to be unnecessary in males in the absence of signs or symptoms of gonorrhea however evidence of the infection in sexual partners in the absence or signs and symp toms should be sought In this regard the male homosexual presents a special problem While the tendency of the infection to pro duce asymptomatic carrier states is greater than previously thought most patients har boring the gonococcus will display sooner or later clinical evidence of infection

In women repeated cultures and FA pro-cedures of carefully selected material from the cervix Skene s glands Bartholin s glands the vagina and the rectum offer the only available means for detecting residual infec tion At least one culture should be obtained from these sites from 7 to 10 days after treat ment If prolonged acting penicillin has been used penicillinase must be incorporated in the culture medium As a precautionary measure in both men and women unpro tected sexual exposure should be interdicted until the criteria of cure have been satisfied Relapse if it occurs will be seen most com monly in the first week after treatment Oc casionally it is found that what appears to be a relapse or treatment failure is a rein fection contracted from a regular sex partner who has not been given treatment or is un discovered for lack of proper epidemiologic studies

An exceptional circumstance is presented when gonorrhea and syphilis or other vene real diseases are contracted concurrently This is by no means a rare occurrence Some 3 per cent of the patients attending VD clinics have acquired syphilis and gonorrhea simultaneously In these instances the gono coccal infection because of its short incuba tion period becomes evident while the syphilitic infection is still in the preclinical or incubation stage Penicillin therapy di rected toward the cure of gonorrhea if in the amount and the type of preparation suggested previously will allow for the aborting of an oncoming syphilitic infection in practically all instances Garson (1963) has proposed a single injection treatment for all acute gonor rhea predicated on the penicillin curative dose for all forms of early syphilis In effect then the treatment of gonorrhea truly becomes a major element in the control of syphilis The occurrence of chills or fever (Herxheimer reaction) accompanying penicillin therapy of gonorrhea is strong presumptive evidence of the coexistence of syphilis as shown by by Fromer et al (1946) Hence all patients who are to receive penicillin treatment for gonorrhea should have a serologic test for syphilis prior to or at the time of treatment and monthly for 4 months after the comple tion of treatment The same precautions should be observed in patients receiving pro phylactic treatment and treatment with other antibiotics

EPIDEMIOLOGY

Reliable information on the incidence and the prevalence of the disease depends on faithful reporting of diagnosed cases As previously indicated it is extremely difficult to diagnose some cases of gonorrhea par ticularly in the female This fact plus the inadequacy of reporting cases makes it prac tically impossible to accumulate any reliable data on prevalence As a consequence figures concerning incidence represent only a por tion of the true picture Although knowledge of the natural history and the course of this disease is considerably better than information

argument but the further assumption that these factors alone would greatly modify the epidemiology of gonorrhea and bring about a marked decline in the prevalence of the disease is not valid The solution of one facet of the problem led to and was largely offset by the increased opportunity for reinfection Because of the short incubation period an increase in the number of reinfections in a given period of time will greatly swell the morbidity statistics and tend to maintain the disease in society Thus the hoped for decline in the prevalence and the incidence of gonorrhea following the introduction of effective antimicrobial agents has not occurred indeed reported cases of gonorrhea have shown a consistent significant upward trend since 1958

Becoming aware of the limitations of gonorrhea control programs the U S Public Health Service in 1952 with the cooperation of State Health Departments and selected large cities in the United States initiated the first major attempt to incorporate known epidemiologic information into a new methodology The technic was called speed zone epidemiology (peppy epi) It is an attempt to apply specialized technics in gonorrhea case finding interviewing and investigation of contacts as well as patient education which will exploit the characteristics of this disease Mahoney *et al* (1946) had demonstrated that the incubation period of experimentally acquired gonorrhea in males has a 31-day range but that 85 per cent of the infections produced clinical symptoms within 6 days Presumably then the bulk of case finding could be narrowed down in at least 85 per cent of all cases to the epidemiologic investigation of sex contacts of gonorrhea patients for the period beginning 6 to 7 days prior to the onset of clinical symptoms and ending at the time of appearance at the clinic If valid information were obtained at interview of the patient with gonorrhea the source case and the spread or contact cases would be identified and hopefully located and brought in for treatment thereby breaking the chain of infection surrounding the particular patient Since at that time penicillin in aluminum monostearate was the longest acting penicillin available and presumably protected the patient from reinfection for at least 72 hours and since reinfection from the same sources without such protection was known to be common and since developed interviewing technics had been proved to be highly effective in identifying contacts investigation of the patient s contacts had to be accomplished within the treated patient s penicillin protective period i e 72 hours Rapid investigation is therefore essential for breaking the chains of infection and protecting the patient from reinfection The most commonly employed technics to bring sex contacts to clinic observation within the 72 hour period were (1) a telegram to the contact requesting immediate appearance at the health department (2) an immediate telephone call to the contact (3) the immediate assignment of the contact not having an adequate address or telephone number or not responding to these technics to a venereal disease investigator and (4) having the informant bring in his contacts within 24 hours of the informant s treatment

Only male patients with diagnosed gonorrhea were interviewed for sex contacts Most well run large clinics had observed that the stimulus supplied to the patient by gonococcal urethritis was sufficient to attain excellent volunteer reporting by males As the females in most instances were completely unaware or belatedly aware of their gonococcal involvement female volunteering could not be depended on Hence adequate case finding among females depended on male cooperation in the identification of sex contacts Occasionally local circumstances will dictate the need for interviewing female cases for contacts The recent successful application of a modification of the syphilis control procedure termed cluster technic has been reported by Brown (1961a) The next essential is the immediate treatment of all sexual contacts brought to the observation of the clinic whether they are diagnosed on observation as gonorrhea or not

Utilizing this technic in a large mid Southern city over a 1 year period it was possible to obtain an index of 1 7 sex contacts per patient interviewed and to bring approximately 85 per cent of the sex contacts to clinic observation and treatment within 72 hours with 50 to 75 per cent of

them There was an almost inconsequential decrease in the rate and the number of cases reported during the 5 years from 1952 to 1956 Beginning in 1957 there has been a continual yearly increase in gonorrhea It has been estimated that the actual incidence of gonorrhea is between 5 and 10 times the number of cases reported (Curtis 1963)

PROPHYLAXIS

Mechanical prophylaxis by means of a condom offers the only type of protection on which any reasonable reliance can be placed In the male transmission of the disease is effected by the contamination of the very distal portion of the urethral mucosa On hypothetical grounds any chemical agent capable of destroying *N gonorrhoeae* while the organisms occupy a vulnerable position on the surface of the mucous membrane should serve as an effective prophylactic However there is no acceptable evidence that the commercial chemical prophylactic preparations or those used in military organizations have any appreciable degree of effectiveness In women mechanical cleansing with soap and water or with mild antiseptic solution offers a theoretical but unconfirmed mode of protection The prophylactic use of penicillin has been explored by the Armed Forces in high incidence areas The utilization of a single oral benzathine penicillin G tablet (250 000 units) is employed in the following ways either on embarking for short term leave or on return from leave 1 penicillin tablet is given to all personnel similarly a tablet may be given on reporting exposure The employment of such a technic on all personnel while indicated under certain circumstances, has been criticized from the standpoint of initiating penicillin hypersensitivity in a certain proportion of the personnel and because of the rather large cost of such a program per gonorrhea case prevented In a well-disciplined group it would appear that the best utilization of this technic would be based on personnel reporting exposure In any event this measure is considerably more effective in the prevention of gonorrhea than the chemical prophylaxis formerly utilized by the Armed Forces

All contacts of patients with gonorrhea should be identified located and treated as soon as possible Of course such contacts should be examined to determine their diagnostic status but regardless of whether the examination indicates the presence of gonorrhea or not all such contacts should be treated because of delays and difficulties in diagnosis particularly in females and the practical fact that sexual exposure usually continues despite advice to the contrary under such circumstances Every effort should be made to include all sexual contacts of the 6 to 10 days prior to the onset of the clinical infection as well as those contacts occurring from the time of onset until the patient is first seen

CONTROL MEASURES

The control of communicable diseases is based upon 4 major principles namely quarantine immunization eradication of the intermediate host, and specific treatment When we attempt to apply these principles to gonorrhea we become more fully aware of the difficulties in the control of this disease The success of the quarantine of cases and contacts depends in large measure on the capacity and the resources available to diagnose cases and find contacts The admitted deficiencies in ability to diagnose this disease particularly among females and the staggering job of attempting to find and process properly and handle perhaps 2 000 000 of our population make quarantine both impractical as well as politically unfeasible We have no means of artificial immunization for this disease and to worsen the situation no effective natural immunity is acquired by having the disease Man is the only known reservoir of gonorrhea and there is no intermediate host to eradicate A special treatment is available but specific therapy alone given to diagnosed cases without the aid of one or more of the other principles of control cannot control a social disease of this nature

It has been assumed that the general use of penicillin therapy would both reduce the period of infectiousness and decrease opportunities for transmission That these assumptions are correct there can be no

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Disease Program, U S Public Health Service is still timely

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the contacts being treated within 24 hours it allowed for a significant reduction in morbidity over the previous year Although not universally successful these programs demonstrate the possibility of rapid investigation and stimulate new and better epidemiologic technics for more practical and economic control

Excellent results of the use of this technic in Canada have been reported by Anderson and Nelson (1954) and Nelson (1957) A most worthwhile and interesting by product of speed zone epidemiology programs has been the rather dramatic reduction in syphilis in the same population groups with no effort being expended other than the usual technics which were in vogue prior to the addition of the gonorrhea program Presumably the significant reduction in syphilis was due to the aborting of incubation or preclinical syphilis as a result of penicillin treatment on a fairly continuous basis

As indicated speed zone epidemiology as a technic for gonorrhea control is hardly a panacea This is understandable when one considers that such programs allow that at least 15 per cent of the contacts named within the jurisdiction of the health department are not located and brought to treatment Other limitations are the occasional failure at interview to obtain any contacts from the patient with gonorrhea contacts outside the jurisdiction of the health agency misinformation given by the informant to cover up his real contacts the undetermined number of unknown cases of gonorrhea in the population at any one time through the failure of the infected individual to seek ethical medical aid

The observations of Hookings (1956 57) on the utilization of combined penicillin aluminum monostearate and benzathine penicillin G in the gonorrhea speed zone project in Memphis Tenn represents perhaps the first breakthrough in increasing the effectiveness of such control programs since their initiation in 1952 On January 1 1956 the Memphis clinic began to treat female contacts and patients with 600 000 units of penicillin aluminum monostearate plus 1 200 000 units of benzathine penicillin G At the same time the records of all female contacts were tagged so that any repeat visit to the clinic would be readily recognized By the end of 60 days the repeat rate which had been 15 per cent in 1955 had dropped to 1 7 per cent A decline was seen in the weekly male attendance at the clinic when compared with the previous 2 year period On April 1 1956 males as well as females began to receive the combined treatment Since that time there has been an increasingly marked decline in male as well as female attendance at the clinic Careful scrutiny of all other factors which could account for such a decline were made and no factor other than the change in the treatment schedules utilized in the speed zone program could be detected Continuation of this approach for the remainder of 1956 allowed for a decrease in morbidity of approximately 1 100 cases over the previous year and also a reduction in total reported syphilis of approximately 50 per cent in 1957

Health department practice calls for

1 Efficient case reporting in order that health organizations may be informed of the trends of prevalence incidence and distribution of the disease

2 Adequate facilities for interviewing prophylactic or diagnostic treatment and newer laboratory diagnostic procedures available freely to the medical resources of the community

3 The rapid investigation of all sources of infection with the bringing to clinic observation during the period of penicillin protection from reinfection of the patient of individuals sexually exposed to a known infected person

4 Informational educational and health promotional efforts both as regards the disease itself and along the broader lines of social hygiene sex education and family living

5 The stimulation of and cooperation with other resources and social agencies within the community whose programs can be effectively brought to bear on the problem

Until the above practices are utilized widely and faithfully we may expect that gonorrhea will continue to be ubiquitous in our society The following statement by Dr C A Smith, formerly Chief of the Venereal

Disease Program, U S Public Health Service is still timely

Venereal disease is unique among the communicable infections in that it is not wholly a clinical problem Its roots lie in the socially unacceptable environment in social maladjustment The venereal disease control program deals with the dilemma that is apparent that is the presence of a venereal disease Our services are specific we treat the infected individual and try to give him enough information so that he will not become reinfected or if he does that he will know what to do about it However we only skirt the edges when we look at the clinical aspects of the disease and do not deal with the deeper social causes or remote origin of the person s present difficulties

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20

The Diphtheria Bacilli and the Diphtheroid

CORYNEBACTERIUM DIPHTHERIAE

Wilson and Miles (1955a) define this group of microorganisms as follows

Gram positive rod like forms arranged usually in a palisade Not acid fast Often with club shaped swellings at the poles generally with irregularly staining segments or granules Nonmotile nonsporing Growing aerobically or under microaerophilic conditions but often capable of anaerobic cultivation Never forming gas in carbohydrate media in which they may or may not produce acidity They may or may not liquefy gelatin or serum Some species produce a powerful exotoxin Type species *Corynebacterium diphtheriae*

Although representatives of the coryne bacteria are widely distributed in nature many strains appear to be associated with the body surfaces and tissues of animals and man Certain species are of significance in veterinary pathology but in human disease the group of closely related forms known collectively as the diphtheria bacilli (*C diphtheriae*) occupy a position of outstanding and unique importance

In many respects diphtheria represents the disease in which the bacteriologist may feel the pride of maximal achievement Most of the facts regarding it—etiology mode of transmission mechanism of pathogenesis therapy and prevention—have been thoroughly elucidated Diphtheria once the leading cause of death among children has virtually disappeared as a disease from civilized communities As stated by Burnet (1953) Other diseases are more important causes of death and some have been just as carefully and extensively studied as diphtheria but no other common disease has been so successfully studied Difficulties in application of our knowledge as well as some remaining lacunae in the facts themselves nevertheless permit the disease to continue in certain regions and occasionally to assume formidable proportions

To a considerable degree the success realized in the understanding and the control of diphtheria results from the circumstance that it is primarily a toxic disorder uncomplicated by any generalized invasion of the tissues by the microorganism It may be considered as providing the pattern for the understanding of a group of diseases such as tetanus and botulism, which closely resemble it in mechanism of pathogenesis and of a further group including scarlet fever and gas gangrene in which specific toxins are responsible for an important ele

* This chapter was originally written by the late J Howard Mueller It is doubtful whether the problems connected with cultivation of the diphtheria bacillus and diagnosis of diphtheria have ever been described so succinctly and so critically and these sections have been left largely as he wrote them It is well to recall that Mueller's pioneer studies on nutrition and toxin production of *C diphtheriae* represented the first truly successful and complete identification of the factors required for growth of a fastidious microorganism and led to studies in the metabolism of pathogenic bacteria that had been virtually impossible previous to his work.

ment of the disease process For these rea sons it seems appropriate to select diphtheria as the first of the infectious diseases to receive detailed consideration

History

Although not established as a distinct clinical entity until the 19th century diphtheria in epidemic form has without question existed since the earliest times Undoubtedly it was often confused with other pathologic conditions affecting the throat, the mouth and adjacent tissues streptococcal or fungal infections Vincent s angina nutritional disturbances such as scurvy and others alone or superimposed on diphtheritic infection must have presented extraordinarily perplexing problems in diagnosis to the early physician In spite of these difficulties certain symptoms uniquely characteristic of diphtheria especially the paralysis of the soft palate with resulting regurgitation of fluid through the nose in attempting to swallow indicate clearly that the disease existed in the 6th century and probably even then had been known for hundreds of years, During the years 1735 to 1740 for example New England and the Middle Atlantic states were ravaged by a throat distemper which from its description was almost certainly diphtheria and may well have caused the death of more than 20 per cent of the entire population under 15 years of age in those regions where it occurred (Caulfield 1939) However it was not until 1826 that the French physician Bretonneau of Tours placed the specific clinical diagnosis of diphtheria on a reasonably firm basis and recognized its infectious nature The diphtheria bacillus was seen and described by Klebs in 1883 in smears from pseudomembranes from the throats of patients with the disease However it was Loeffler who a year later established the diphtheria bacillus as the etiologic agent and reproduced a similar disease in animals using the bacterium grown in artificial culture For this reason the diphtheria bacillus has often been referred to as the Klebs Loeffler or K L bacillus Loeffler made the important observation that both in fatal human cases and in laboratory animals the organisms could rarely if ever be demonstrated except in the local lesion of the mucous membrane Although tissue damage occurred in many remote organisms such as adrenals liver and heart these invariably proved to be sterile This led Loeffler to postulate the formation of a diffusible poison by the organisms His prediction was verified in 1888 when Roux and Yersin announced the discovery of diphtheria toxin Roux and Yersin demonstrated that injection of sterile culture filtrates of the diphtheria bacillus into laboratory animals caused a fatal disease with lesions similar to those produced by living organisms Two years later von Behring and Kitasato found that repeated sublethal doses of toxin partially detoxified with iodine trichloride caused animals to elaborate a substance antitoxin which was capable of specifically neutralizing the toxin By 1891 sera from immunized animals were being used in the treatment of diphtheria Thus the foundation was laid for a half century of specific serum therapy and prophylaxis not only of diphtheria but also of a variety of other infectious diseases

The methods for controlling diphtheria in populations by mass immunization followed logically from these early observations and from the development of quantitative methods for bio-assay of toxin and antitoxin by Paul Ehrlich

Suggested by Theobald Smith in 1909 toxin neutralized by antitoxin was shown by Behring in 1913 to induce immunity safely in both animals and man and was applied on a large scale by Park (1922) for the protection of children A simple test for immunity by the intracutaneous injection of minute amounts of toxin was developed in 1913 by Schick making it possible to define more accurately the need for and the results of artificial immunization Finally Ramon in 1923 showed that formalin treated toxin *anatoxin* (now commonly called *toxoid*) possessed certain advantages as an immunizing agent over toxin antitoxin mixtures and this material in one form or another has been used for wholesale immunization especially of children in many communities Thus the knowledge and the tools appear to be at hand for eradicating diphtheria and indeed the disease has already become uncommon in most civilized countries

The most recent chapter in the history of diphtheria was opened by Freeman's discovery in 1951 that only those bacterial strains growing in close association with a particular temperate bacteriophage are capable of producing diphtheria toxin Strains which do not carry the virus are nontoxinogenic

MORPHOLOGY

The characteristic feature of the coryne bacteria from which they derive their name is their varying diameter, often broader at one end than the other resulting in a club shape A further characteristic depends on the nonuniform absorption of certain dyes resulting in a beaded or barred appearance of the organisms when suitably stained This may be particularly noticeable in the case of certain strains of the diphtheria bacillus often accentuated by growth on special media and leading to the appearance of well defined polar bodies These deeply staining bands and beads have been variously named metachromatic granules Babes Ernst bodies etc and are composed mainly of high molecular weight polyphosphates (Ebel 1952) Corynebacteria are gram positive non spore bearing rods without flagella or capsules which vary in size from 2 to several micra in length and from 0 5 to 1 0 micron in diameter Evidently because following cell division the two resulting bacteria break apart sharply the distribution of organisms in a stained smear is relatively characteristic The individual bacilli form sharp angles with each other and have been variously compared with piles of matches Chinese letters or cuneiform characters to which the frequent wedge shape of the cell lends further suggestion The occasional occurrence of true branching which has been observed in the growth of *C diphtheriae* together with the irregularities of protoplasmic distribution previously mentioned have been interpreted by some bacteriologists as tending to separate this group of organisms from the true bacteria and place it somewhat above them in the organizational scale

The keynote of microscopic morphology of the Corynebacteria is variability not only between strains but even within a given strain grown under different conditions Nevertheless the microscopic appearance of this group of organisms is sufficiently distinctive to render them among the most easily recognized of all the human pathogens (see Fig 1)

While morphology can aid the bacteriologist in the laboratory diagnosis of diphtheria and in the detection of carriers final identification always must rest on further characterization of cultural and biologic properties of the organisms The existence of nontoxinogenic diphtheria bacilli which are morphologically and culturally identical with toxinogenic strains serves to underline the limitations of morphologic criteria in diagnosis

For many years it has been known that potassium tellurite in amounts that inhibit the growth of most bacteria has little effect on *C diphtheriae* For this reason an agar medium containing tellurite provides the bacteriologist with a reasonably selective means for isolation and diagnosis Using tellurite



FIG 1 Three different strains of *Corynebacterium diphtheriae* stained with Loeffler's methylene blue (× 760)

agar Anderson *et al* (1931) were able to differentiate clearly between two colonial types of *C diphtheriae* which they isolated from humans during an unusually severe outbreak of diphtheria that occurred in England

The two main types give rise to *rough* and *smooth* colonies In general the rough forms are the largest tend to be flat slate gray to black in color with a dull or matt surface Smooth colonies are usually blacker convex with a smooth glossy surface and somewhat smaller It was thought at first that the rough strains were associated with a more severe form of diphtheria with a higher death rate than that caused by smooth strains For this reason the types were originally named *gravis* and *mitis* It now seems certain that no simple relationship exists between colonial morphology and clinical severity for example strains can be isolated which are rough with typical *gravis* morphology yet produce no toxin at all Therefore it seems best to abandon the older terminology and simply refer to the two main types as rough and smooth A third type giving rise to dwarf colonies which may be either smooth or rough has been termed *minimus* (Frobisher 1940) or *intermedius* (McLeod 1943) It produces minute pinpoint colonies that vary from gray to black depending on the particular formula of tellurite medium used It now seems certain that the various types can be derived from one another by mutation Colonies of *C hofmannii* and some strains of staphylococci resemble the smooth type of *C diphtheriae* in size and color but are usually sufficiently different to permit recognition by the experienced bacteriologist

In broth rough strains of *C diphtheriae* tend to grow in a pellicle on the surface of the medium whereas smooth strains grow more diffusely The *dwarf* or *minimus* strains develop as a finely granular turbidity in broth settling to leave a clear supernatant

Diphtheria bacilli may be divided into groups according to their antigenic composition Doubtless because toxinogeny is by far the most important factor concerned in pathogenicity erologic classification of this group of organisms has not received intensive study Up to the present no clear-cut relationship of immunologic properties to the main mutant types has been found

It is evident from the above that the nature of growth to be expected of a diphtheria bacillus in the usual bacteriologic bouillon the agar slope or plate blood agar plates etc will vary with the type of the organism and that no further attempt at detailed description would be profitable

Cultivation

C diphtheriae is primarily an aerobic organism and multiplies poorly if at all under strict anaerobic conditions It grows readily on most of the usual laboratory media containing peptones and tissue extractives On liquid medium most strains tend to grow as a waxy pellicle or veil on the surface The nutritional requirements have been thoroughly investigated by Mueller (1940) who has shown that most strains require a variable number of specific amino acids and a carbon or energy source which may be glucose or some other sugar an organic acid or alcohol In addition most strains are deficient in their ability to synthesize biotin nicotinic acid and pantothenic acid Under the optimal conditions of oxygen supply pH control etc used in toxin production growth equivalent to 20 or more Gm dry weight can be attained per liter of liquid medium

For initiating growth from minute inocula for example on agar plates traces of oleic acid and some additional unidentified substance have been shown to be necessary (Cohen and Mueller 1941)

For primary isolation of the diphtheria bacillus a number of tellurite-containing agar media have been proposed as mentioned in the preceding section (*Mueller and Miller* 1946) The classic medium still used in bacteriologic diagnosis is the coagulated blood serum slant first described by Loeffler The belief has arisen that Loeffler s medium possesses selective growth promoting properties for the diphtheria bacillus enabling it to outgrow other bacteria occurring in the throat and giving it a normal and typical morphology While there is no question of the utility of Loeffler s medium for diagnostic purposes it seems in the light of present knowledge that its success is due largely to

the fact that it is a relatively poor medium on which the diphtheria bacillus grows reasonably well but is not quite good enough for the average streptococcus or pneumococcus Perhaps it is worth noting that rather widely divergent results will be obtained on Loeffler s medium both as regards excellence of growth and morphology depending on the species of serum used in its preparation Beef serum is most commonly employed

Bacteriologic Diagnosis

Current views tend to place the responsibility for diagnosing diphtheria on the clinician leaving for the laboratory the task of bacteriologic confirmation This practice has been adopted because of the risk to the patient of delay in the administration of antitoxin It is safer to err on the side of an occasional needless serum treatment than to lose time which can make the difference between recovery and death To a degree therefore the necessity for a very rapid diagnosis need no longer be felt by the bacteriologist although the diagnostic method employed should be no more time-consuming than is consistent with accuracy The method to be selected must also be readily applicable to recognition of the diphtheria bacillus in the convalescent case and the healthy carrier as well as from the occasional unusual source such as the conjunctiva a skin lesion or wound diphtheria

The diagnosis depends on the recognition of a diphtheria bacillus in material taken from the site of infection usually the throat It is important that the specimen be obtained carefully and that it represent material from the membrane if present and from the area of inflammation It should be taken by the physician with good illumination of the area and sent to the laboratory with the least possible delay While the diphtheria bacilli are not especially delicate and may survive for many hours on a cotton swab they die out progressively and the interval between taking the culture and its examination should be no greater than necessary In the laboratory a Loeffler slant a blood plate and a tellurite plate are inoculated with the swab and a smear is prepared which may be stained with dilute fuchsin and examined for the organisms of Vincent s angina It is not recommended that any attempt be made to identify *C diphtheriae* in the direct smear Except in the hands of a bacteriologist of long experience in such diagnosis, the chance of error in either direction is too great After 15 to 24 hours incubation the cultures may be examined—the blood plate for hemolytic streptococci and the tellurite plate for the gray or black colonies of diphtheria bacilli Frequently no growth whatever will be found on the tellurite plate Provided that the blood plate has shown the presence of viable bacteria on the swab this furnishes strong presumptive evidence that no diphtheria bacilli are present but the plate should be reincubated for 24 hours more and again examined before being reported finally as negative In examining the tellurite plate one must keep in mind the different appearance of the types of *C diphtheriae* and especially the relatively inconspicuous nature of *intermedius* or *minimus* growth If colonies are present which suggest any of the types, a smear stained with methylene blue will enable one to determine promptly whether or not they are corynebacteria of some sort If so their morphology together with the colony appearance will frequently make identification quite certain In case of doubt the somewhat different morphology of the organism on the Loeffler slant may be of assistance *C hofmannii* for example is sufficiently characteristic to make recognition relatively easy

Where considerable numbers of cases of infection due to a single type (i e *rough smooth* or *dwarf*) of *C diphtheriae* are occurring in a community the matter need be carried no further the bacteriologist will readily identify the strain and a final report may be made covering the presence of the diphtheria bacillus the hemolytic streptococcus and Vincent s angina

Organisms from the suspected individual case the possible carrier (except during an epidemic of a known type) perhaps the occasional refractory convalescent carrier and from any other unusual source such as a skin lesion should be isolated for further identification and a test for toxinogeny carried out

It is usually desirable to restreak a second tellurite or blood agar plate from the growth on the initial tellurite plate and then to isolate the culture from a single colony to a Loeffler slant or other suitable medium The pure culture thus obtained is used to inoculate tubes of Hiss serum water to check the fermentative properties Ordinarily glucose maltose and sucrose will be found to be sufficient for this purpose Failure to ferment a sugar should be recorded only when demonstrable growth has occurred Table 1 summarizes the fermentation reactions of *C diphtheriae* and of certain related corynebacteria frequently encountered in the nasopharynx or the throat of man

The test for toxinogeny (so called virulence test) may be carried out in the following manner

The growth on Loeffler slants is emulsified with broth to give slightly turbid bacterial suspensions From 0 1 to 0 2 ml of each suspension is injected intradermally into one (shaved) side of a guinea pig Then the suspensions are placed in the refrigerator After 4 hours 500 units of antitoxin is administered intraperitoneally and 30 minutes later the test suspensions are injected into corresponding sites on the other side of the guinea pig Inflammatory reactions may occur at both sites within 24 to 48 hours but only sites injected with a toxinogenic strain *before* antitoxin was administered go on to develop characteristic necrotic lesions at 48 to 72 hours This method of testing for toxinogeny has the advantage that lesions caused by a single bacterial suspension before and after injection of antitoxin may be compared in the same animal In case it is necessary to test many isolates rabbits may be used and are equally satisfactory

Some nontoxinogenic strains are capable of causing pyogenic local skin lesions which resemble staphylococcal infections in rabbits and guinea pigs Similar lesions may be observed with certain toxinogenic strains even in control animals previously given antitoxin However such pyogenic lesions are easily differentiated from the larger hemorrhagic and necrotic lesions produced by toxinogenic strains in the test animals

A convenient in vitro method of testing for toxinogeny was described by Elek

TABLE 1

	GLUCOSE	MALTOSE	SUCROSE
C diphtheriae (most strains)	+	+	−
C diphtheriae (some strains)	+	+	+
C hofmannii	−	−	−
C xerosis	+	−	+

(1948) based on Ochterlony s technic for detecting precipitin reactions by double diffusion in agar gels More recent modifications of the method by Hermann *et al* (1958) and Liu (1961) permit the reaction to be carried out on microscopic slides on which positive reactions develop within 12 to 18 hours

PATHOGENESIS

Diphtheria is the classic example of an infectious disease in which the causative organism is capable of only limited invasion of superficial tissue and in which almost all of the severe damage to cells may be attributed to a soluble toxin released by the bacteria and carried to remote organs by the bloodstream In man the site of the local lesion is most commonly the throat or the nasopharynx It is true that certain non toxin producing strains of *C diphtheriae* are capable of producing a mild sore throat and fever of short duration in human subjects (Edward and Allison 1951) Such strains may produce purulent local lesions when injected intradermally into rabbits However the classic severe disease is *always* caused by toxinogenic strains To understand the pathogenesis of diphtheria we must (1) explain how the organisms become established in the local lesion (2) describe conditions under which toxin is synthesized and released by the diphtheria bacillus and finally (3) elucidate the nature of the toxin molecule and the mechanism by which it kills the susceptible host

The capacity of the diphtheria bacillus to cause disease evidently arose through a number of improbable mutational events Thus only those strains of *C diphtheriae* that are lysogenic for the bacteriophage β or for certain mutants of β are toxinogenic Nonlyso

genic strains are nontoxinogenic and therefore incapable of causing typical diphtheria. Moreover, even in the presence of β prophage, synthesis and release of toxin occurs only under particular abnormal conditions in which the bacterial iron content and complement of iron-containing respiratory enzymes is being depleted.

Toxinogeny and Lysogeny. It was shown by Freeman (1951) that when nontoxinogenic strains of *C. diphtheriae* are treated with a particular bacteriophage, a certain proportion of the resistant survivors on plating give rise to colonies of toxinogenic bacteria. These survivors are lysogenic: they carry a "latent virus," the prophage β. In cultures of the converted toxinogenic organisms a small proportion (about 1 in 50,000 cells) spontaneously lyses to release mature β phage which can then infect and convert new sensitive cells to toxinogeny and lysogeny. Following irradiation of a lysogenic culture with ultraviolet light, multiplication of vegetative phage is induced, and after about two generation times most of the bacteria lyse with release of about 30 phage particles per bacterium.

When the temperate phage β is added to a sensitive culture and its DNA is injected into a cell, one of three things may happen. (1) The injected DNA fails to integrate with the host genome, does not replicate, and is lost on cell division. The progeny once more become sensitive. (2) The DNA replicates, vegetative phage is synthesized, and the cell bursts with release of 30 β particles per cell, or (3) the injected DNA becomes integrated with the host genome and the cell and its progeny become toxinogenic and lysogenic. The studies of Groman (1953) and of Barksdale and Pappenheimer (1954) suggest that *all* toxinogenic diphtheria bacilli are lysogenic for β phage or one of its mutants.

Groman (1955) has shown that "cured" diphtheria bacilli from which β prophage has been eliminated are no longer able to produce toxin. The cured strains are again sensitive to β phage and lysogenization of them again results in toxinogeny. This type of alteration of a bacterial genome brought about by the mere presence of a particular prophage is known as *lysogenic conversion*. This phenomenon differs from *transduction*, where the bacterial genome becomes altered by the introduction of new *bacterial* genetic material through the agency of phage. The acquired property in the case of transduction is controlled by bacterial genes, whereas in lysogenic conversion the new attribute is apparently under the control of prophage.

Other diphtherial phages exist which are capable of lysogenizing without converting sensitive strains to toxinogeny. Groman *et al.* (1958) have formed doubly lysogenic strains which carry both toxinogenic and nontoxinogenic prophages β and γ, respectively. Following induction of such doubly lysogenic organisms, they succeeded in isolating recombinant phages, β' which had lost its ability to convert sensitive bacteria to toxinogeny, and γ' which had acquired this property. The host ranges of β' and γ' remained the same as those of the parental β and γ phages. Thus not all mutants of β phage are capable of converting to toxinogeny.

Although the capacity of a given strain of *C. diphtheriae* to produce toxin requires the presence of a suitable mutant of β phage, whether or not toxin is actually synthesized and in what yield are determined by the host bacteria's own metabolism. As will be discussed in the following section, even when β phage is present in a given organism, toxin is produced only under certain unfavorable conditions of growth. Little or no toxin is synthesized by toxinogenic lysogenic strains during normal exponential growth.

Production and Properties of Diphtheria Toxin. Since the clinical injury caused by *C. diphtheriae* is almost entirely accounted for by the toxin which it forms, and since control of the disease depends on obtaining high yields of toxin for conversion to toxoid, the production and the properties of this substance have practical as well as theoretic interest. In order to obtain high yields of toxin in artificial media, it is first essential to select a suitable lysogenic strain. In 1898 Park and Williams isolated from a mild case of diphtheria an atypical slow growing strain which they found produced an exceptionally potent toxin. In fact, no other strain has ever been isolated that is capable of producing such high yields of diphtheria toxin as the PW8 strain. Under suitable conditions, the differential rate of synthesis of toxin by this strain

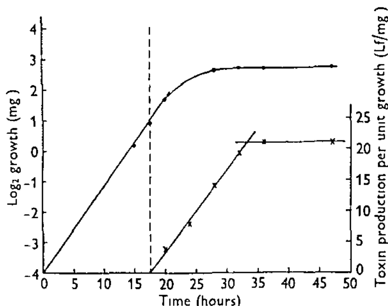


FIG 2 Toxin production and growth of the PWB strain of *C diphtheriae* as a function of time Crosses toxin production per unit growth (Pappenheimer A M Jr 1955 *in* Mechanisms of Microbial Pathogenicity Cambridge University Press)

is equivalent to 5 per cent or more of the total bacterial protein For this reason the PW8 strain is used in almost all laboratories throughout the world for the routine production of toxin

The conditions for obtaining maximal growth of the PW8 strain a necessary prerequisite for obtaining high yields of toxin were worked out by Mueller and Miller (1940) and Drew and Mueller (1951) More recently toxin has been obtained in unusually high yields by growth in submerged cultures in which temperature pH and aeration are continuously controlled (Linggood *et al* 1955) Up to 20 Gm per liter dry weight bacteria can be harvested and the culture filtrates contain up to 300 Lf/ml toxin (equivalent to nearly 1 gram per liter of toxic protein)

The most important single factor controlling toxin production is the iron content of the culture medium (Pappenheimer and Johnson 1936) As shown in Figure 2 no toxin is formed in well aerated shake cultures during exponential growth Only after the iron content of the medium has become exhausted is toxin formed in significant amounts Its release continues at a linear rate until bacterial growth ceases In the case of the PW8 strain the bacterial mass increases 4 to 6 fold during toxin production At the same time the cellular content of iron-containing respiratory enzymes (cytochromes) falls to ¼ to ⅙ and equivalent amounts of free coproporphyrin III and toxin are liberated into the culture medium At no time are more than traces found in or on the cells The unusually high yield of toxin produced by the PW8 strain appears to be related to its ability to grow at a reduced cellular iron content and not to any peculiarity in the prophage which it carries Recent studies (Pappenheimer Howland and Miller 1962) have shown that in contrast with most naturally occurring diphtheria bacilli the PW8 strain contains a defective and incomplete cytochrome system and uses flavoprotein rather than cytochrome as terminal oxidase Other strains of the diphtheria bacillus although they contain a complete cytochrome system are unable to grow once their iron content has been reduced by more than 30 to 40 per cent (compared with 80 to 85% for PW8) and the maximum yields of toxin are correspondingly reduced

Diphtheria toxin was first isolated in crystalline form by Pope and Stevens (1958) (see Fig 3) It is a heat labile protein of molecular weight 72 000 and is homogeneous by all of the usual physicochemical criteria Chemical analysis of the crystalline protein has revealed no groupings other than

the usual amino acids The most active preparations are lethal for susceptible animals in doses of less than 0 1 mcg /Kg

The relationship between lysogeny and toxinogeny has remained obscure Studies on the kinetics of toxin synthesis by bacteria labeled with radioactive amino acids have shown that the toxic protein is synthesized *de novo* by cells of decreasing iron content and that its release is not accompanied by lysis of any significant number of cells (Pappenheimer Miller and Yoneda 1962) Indeed during its production toxin may account for 75 per cent or more of the total extracellular protein There is no convincing evidence that phage multiplication is involved in toxin production

In summary then although diphtheria toxin has been characterized as a protein and the kinetics of its synthesis and release by lysogenic strains of decreasing iron content are reasonably well understood we still do not know what toxin is or why it is formed by bacteria Because of the close quantitative relationships between toxin and coproporphyrin III release on the one hand and bacterial iron and cytochrome contents on the other the suggestion was made (Pappenheimer and Hendee 1947 Pappenheimer 1955) that the toxin may be related structurally to the protein moiety of a cytochrome *b* the major respiratory pigment of the PW8 strain

Pathogenicity of C Diphtheriae for Animals and the Mode of Action of Diphtheria Toxin Natural infection of the lower animals with *C diphtheriae* appears not to occur However a variety of experimental animals are susceptible to the effects of its toxin and it has been possible to establish with the organism experimental infections which simulate the human disease Thus Loeffler in his early experiments was able, by intratracheal inoculation so to infect rabbits and pigeons as to obtain typical diphtheritic pseudomembranes at the site of injury

Injection of diphtheria toxin subcutaneously into an animal such as the guinea pig is followed in 10 to 12 hours by local swelling and apparent tenderness Death ensues in from 12 hours to several days depending on the amount of toxin given With suitably gauged sublethal doses late paralyses may occur resembling those seen in man during convalescence from diphtheria Examination at autopsy following death from a fatal dose reveals intense edema of the subcutaneous tissue at the site of injection often hemorrhagic in character Beyond this the most



FIG 3 Diphtheria toxin (*left*) recrystallized from phosphate buffer (*right*) recrystallized from ammonium sulfate (Pope C G and Stevens M 1958 Brit J Exp Path *39* 142)

constant and striking pathologic picture is the marked congestion of the adrenal cortices frequently accompanied by hemorrhage Occasionally hemorrhage in the pericardium or the diaphragm occurs and the heart muscle the liver and the kidneys may show fatty degenerative changes By definition a minimum lethal dose (M L D) is that amount of diphtheria toxin that will kill a 250 Gm guinea pig on the 4th or the 5th day following subcutaneous injection

If cultures of virulent diphtheria bacilli are injected rather than toxin itself the chain of events and the pathologic findings follow closely those observed after the administration of the toxin The bacilli themselves as Loeffler recognized remain localized and in general are found at autopsy only in the vicinity of the site of inoculation Positive cultures occasionally obtained from the viscera appear to be due to terminal or post mortem invasion rather than attributable to any active invasive ability on the part of the organism

In the diphtheria bacillus therefore we have an organism possessing in some degree the ability to survive and to establish itself in the healthy tissues of a susceptible animal to an extent sufficient for the elaboration of an amount of toxin which is capable of causing death of the animal through specific and distant action on certain kinds of tissue

Within any single given species in the absence of specific antitoxic immunity there is a remarkable uniformity in sensitivity to diphtheria toxin from one individual to the next However different species may vary enormously in their sensitivity Thus although susceptible human beings monkeys rabbits guinea pigs and even pigeons all appear to be equally sensitive when toxicity is expressed as lethal doses per unit weight rats and mice are several thousand times more resistant The resistance of rats and mice cannot be due to the presence of substances that neutralize or inactivate toxin since the serum of rats injected with diphtheria toxin remains toxic for guinea pigs for several days It is possible that some kind of permeability effect may account for the resistance of rat and mouse cells since the same type of tissue damage occurs as in guinea pigs provided that a sufficiently high dose of toxin is given A similar explanation may account for the fact that cold blooded vertebrates such as amphibia and reptiles are resistant to toxin at lower temperatures but become susceptible when their body temperature is raised above 25 or 30° C (Grasset and Zonderdyk 1931)

For many years it was thought that only vertebrates were susceptible to the action of diphtheria toxin However as will be discussed below it was shown by Pappenheimer and Williams (1952) that insects such as the cecropia silkworm are extremely sensitive to the toxin at certain stages in their metamorphosis Other lower forms such as protozoa yeasts and plants appear to be wholly resistant

Recently mammalian cell lines growing in tissue culture have been used as experimental animals The so-called spinner cultures of sensitive established lines such as HeLa cells have proved to be particularly useful in studies on the mode of action of the toxin because they provide a uniform population of cells growing in suspension It is of interest that cell lines derived from mouse and rat tissues are resistant to the toxin whereas those of human, monkey or guinea pig origin are highly sensitive (Lennox and Kaplan 1957 Gabliks and Solotorofsky 1962) Thus the sensitivities of the cells in culture reflect those of the animal from which they were derived

In attempts to discover the mode of action of diphtheria toxin it has proved to be difficult to distinguish between primary and secondary toxic effects Even when large doses of toxin are injected into animals there is a latent period of several hours during which no symptoms or evidence for morphologic or biochemical damage can be detected Changes that cannot be observed until after the latent period are likely to prove to be secondary to some earlier and more subtle injury Very few molecules of the toxic protein are required to kill a cell In fact it can be calculated from the studies of Lennox and Kaplan and Gabliks and Solotorofsky that highly sensitive mammalian cells are killed when the tissue culture medium in which they are grown contains a total of only 200 to 300 molecules of toxin per cell At such low concentrations (about 10^{13} M) there is a latent

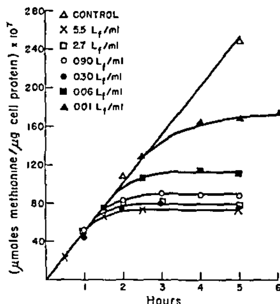


FIG 4 Effect of increasing concentrations of purified diphtheria toxin on the incorporation of methionine into protein by growing HeLa cells (*Strauss* N and Hendee E D J Exp Med *109* 145)

period of 48 hours or more before the first signs of morphologic damage can be detected A similar prolonged latent period is observed when small doses are injected into animals Such extreme toxicity seems to preclude the likelihood of injury by direct fixation of toxin to a stable cell component and suggests that the toxin must act catalytically to activate or to inactivate some vital enzyme or other cell catalyst

In the preceding section evidence suggesting a possible relation between toxin and diphtherial cytochrome was discussed This hypothesis led in turn to speculation that toxin might interfere in some manner with cytochrome linked energy metabolism in the susceptible animal At first experiments appeared to be in keeping with such a hypothesis Thus Pinchot and Bloom (1950) demonstrated a depletion of phosphocreatine and decreased muscle organic phosphate in intoxicated guinea pigs In their studies on the cecropia silkworm Pappenheimer and Williams (1952) found that only those tissues containing a complete succinoxidase and cytochrome system were sensitive to the action of the toxin During the pupal diapause of this insect protein synthesis is minimal and a complete cytochrome system is present and functioning only in a small group of intersegmental muscle tissues Despite the destruction of these muscles within a few days, the animal as a whole is not killed by large doses of toxin On the other hand adult development a process that involves rapid synthesis of new protein and a high energy metabolism is brought to a prompt stand still by injection of small amounts of toxin and at this stage the intoxicated insect succumbs within a few days

The most recent studies on the mode of action of diphtheria toxin have used established mammalian cell lines as an experimental tool Strauss and Hendee (1959) found that one or more saturating doses" (0 5 mcg /ml or greater) of toxin completely arrest growth and protein synthesis in cultures of HeLa cells within 1 5 to 2 hours at 37° C although morphologic damage does not become evident until several hours later Far smaller concentrations of toxin cause a similar effect after a more prolonged latent period of normal cell growth Figure 4 shows that incorporation of S^{35} methionine into HeLa cell protein ceases within 2 hours in the presence of toxin concentrations of 0 3 Lf per ml (about 1 mcg / ml or greater) The effect may be prevented by antitoxin added within the first hour The studies of Strauss and Hendee and more recently of Collier and Pappenheimer (1964) *have shown that protein synthesis does not* come to a halt because of a derangement in the energy metabolism of the cell Even after 5 to 6 hours when morphologic damage first becomes detectable the ATP and the high energy content remain normal in intoxicated cells Moreover it has been shown that very low concentrations of toxin ($\geqq$ 0 5 mcg / ml) block incorporation of amino acids into protein by cell free extracts of HeLa cells provided with an ATP generating system and containing ribosomes soluble enzymes and cofactors

We may conclude that while some progress has been made toward the elucidation of the mode of action of diphtheria toxin at the molecular level much remains to be learned

It is certain that toxin in low concentration rapidly arrests protein synthesis and growth of susceptible cells However the exact mechanism by which toxin interferes with amino acid incorporation into protein remains to be solved and indeed it is still uncertain at present whether the inhibition of protein synthesis is a primary or a secondary effect of the toxin

Diphtheria in Man

The diphtheria bacillus occurs in nature so far as is known only in lesions of the specific disease in man and in the throats and the noses of the normal human carrier From one of these two sources a virulent diphtheria bacillus reaches more or less directly—by droplet contact or fomites—the throat of a susceptible individual Growth presumably is initiated in a superficial layer of mucus and desquamated epithelial cells and small amounts of toxin are elaborated This toxin absorbed into adjoining living cells destroys them in a few hours through its local necrotizing action The nidus of necrotic tissue supplies favorable conditions for further growth of the organisms more toxin is formed and the process extends both laterally and more deeply into the tissue

Meanwhile there is an inflammatory reaction on the part of the body capillaries engorge leukocytes enter red cells become extravasated and a layer of exudate begins to form which is composed of all these various elements At first this is grayish and inconspicuous but as the process continues it soon becomes thicker and tough forming a dull white layer or pseudomembrane covering the area The initial lesion may cover a tonsil or a portion of the posterior pharynx In some cases it is limited to the posterior nares or the trachea and thus may elude observation At this stage of the disease the patient has typically a moderately sore throat lacking the acutely inflamed appearance swelling and pain of a streptococcus infection and a relatively low fever of from 100 to 102 F However he usually manifests a degree of prostration out of all proportion to the fever and the visible difficulty in the throat The membrane may spread with considerable rapidity over the tonsils the uvula and the posterior pharynx The dull white color gives place to a dirty gray and later to brown or in some instances black as a result of hemorrhage Separation of the membrane by mechanical means during the early stages uncovers bleeding points and is rapidly followed by the formation of fresh exudate

The cervical glands early become swollen and tender and in the severe or bullneck variety there is a massive edema of the tissue of the neck and the chest If the membrane developed initially in the larynx or if it extends to that site and continues further into the trachea death may result from mechanical stoppage of the air passage unless promptly relieved by intubation or tracheotomy Excluding such mechanical termination of the infection and in the absence of antitoxin treatment the patient will run the natural course of the disease and die during the acute stage as a result of general toxic effect succumb after a somewhat longer time as a result of cardiac damage by the toxin or recover after perhaps showing evidence of neurotoxic injury such as paralysis of the soft palate the ciliary muscles of the eye or the extremities The membrane separates after a few days and is eliminated leaving as a rule very little ulceration of the underlying tissues

The infection may occur initially in the ear or in the anterior nares and in the latter site particularly is likely to be relatively mild though prolonged We have seen a fatal case in a child whom the membrane was located in the umbilicus Rarely the conjunctiva may become the site of extremely severe and destructive diphtheritic lesion

Skin or wound diphtheria is rarely seen under ordinary conditions in temperate climates In the tropics however it is apparently not uncommon and indeed presented the armed forces with a rather serious problem in Africa and in portions of the Pacific theater during World War II The infection appears usually at the site of some relatively minor injury a bruise a scratch or a blister and develops as an ulcer showing little tendency to heal with sharply demarcated edges and a dirty grayish slough or membrane covering the base Such infections may last for weeks or months and cover areas of several centimeters diameter Their nature may be unsuspected because of the rarity of the con

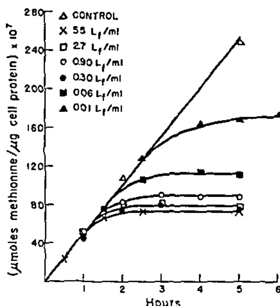


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theria would be 100 per cent Since 1 unit of antitoxin will neutralize (in the test tube) about 30 M L D it might appear that from 100 to 1 000 units would be more than adequate to obtain the desired effect However since it is known from experiments such as those mentioned above that within limits increasing the antitoxin will save life even after contact of the toxin with the tissue there is a reasonable basis for the current practice of treating diphtheria with relatively large doses of serum The objective should be to counteract as much as possible of the injury already done and to prevent further absorption of toxin Antitoxin is eliminated slowly so that a large single dose raising the blood level as high as possible will assure the maximum immediate therapeutic effect and provide a level in the blood for many days which will be adequate to cope with further toxin as it is absorbed slowly from the local process in the throat

Various figures for the recommended unitage of antitoxin are to be found in the literature Perhaps one may state the general average as suggesting 100 units per pound body weight in mild cases up to 5 times that amount in the severe forms as adequate therapy It should be administered in a single dose intravenously except in the very mild case where intramuscular injection is satisfactory Of course skin tests for sensitivity to the protein of the antitoxin preparation must be carried out and where necessary desensitization must be undertaken

While the diphtheria bacillus is highly sensitive to the action of penicillin and other antibiotics chemotherapy is probably of little therapeutic value provided that antitoxin has been administered Once the patient has received antitoxin production of additional toxin by the organisms can probably do no further harm However it is possible that early administration of antibiotics might shorten the duration of the carrier state that almost invariably follows recovery from clinical diphtheria

The Diphtheria Carrier

Patients convalescing from diphtheria continue to harbor the organisms in the nose or the throat for a variable length of time following recovery The accompanying table

TABLE 3 PERCENTAGE OF CLINICAL CASES CARRYING DIPHTHERIA BACILLI DURING WEEKS OF CONVALESCENCE

Week of Convalescence	Nose or Nose and Throat (1 240 Cases)	Throat (1 726 Cases)
0	100	100
2	76 0	50 1
3	63 1	35 3
4	48 3	20 6
6	24 6	7 6
8	12 6	2 6
10	4 8	0 7

modified from Russell (1943) shows that diphtheria bacilli can be isolated from the nose or from the nose and the throat in nearly 50 per cent of the cases during the 4th week of convalescence and that the bacilli are still present in a significant proportion during the 10th week The table suggests that an appreciable number of carriers will be missed if only throat cultures are tested for diphtheria bacilli

The treatment and the disposition of these chronic carriers becomes then a most troublesome problem both scientifically and administratively and one for which no satisfactory solution has been found In many communities the health regulations require a continuation of quarantine of the individual until negative throat cultures are obtained on 3 consecutive days as a result considerable hardship is suffered by the patient and the hospital also may find itself in a difficult situation Experience has shown that surgical correction of nose and throat abnormalities tonsillectomy etc will result in some instances in clearing up the condition At present evidence is accumulating that combined treatment of the case with antitoxin and penicillin in which the administration of the latter is continued for a time into convalescence leads to a much more rapid disappearance of positive cultures than was formerly true Opposed to this is the fact that some observers find diphtheria bacilli reappearing in the throat within a few days after interrupting the penicillin therapy It is therefore still premature to assume that a permanent cure for the convalescent carrier problem has been achieved

TABLE 2

ANTITOXIN GIVEN ON	CASES	CASE FATALITY
1st day of disease	225	0
2nd day of disease	1 441	4 2
3rd day of disease	1 600	11 1
4th day of disease	1 276	17 3
5th day of disease and upward	1 645	18 7

dition under normal circumstances and in temperate climates

It is well recognized that some strains of *C diphtheriae* are capable of causing a more severe disease in man than are other strains In the most severe form of the disease the bacteria presumably produce a relatively large amount of toxin within a short time and death may occur within 24 to 48 hours *after the onset of symptoms*

TREATMENT

Since diphtheria is essentially a toxemia with very little invasion of the tissues by the organisms themselves it appears that prompt recovery should follow administration of the specifically neutralizing antitoxin Unfortunately the problem is not quite so simple Diphtheria toxin in the test tube it is true, is neutralized promptly completely and multiple for multiple by antitoxin Although it can be shown that the toxin is not actually destroyed the union is a relatively firm one and dissociation apparently does not occur to a significant degree if the complex is injected into the animal body However free toxin injected into the circulation is rapidly taken up by body cells and can no longer be neutralized by antitoxin The train of events which the toxin is capable of initiating continues even in the presence of a considerable excess of antitoxin in the circulation Thus if a series of animals be injected with similar quantities of diphtheria toxin the animals being divided into groups and varying amounts of antitoxin administered immediately to one group after 5 minutes to a second group and so on it is found that the amount of antitoxin necessary to save life increases very rapidly with the time interval The following tabulation cited by Zinsser *et al* (1939) shows the amounts of antitoxin necessary to prevent death in rabbits at various intervals after the administration of 10 fatal doses of toxin It is evident that after a delay of 90 minutes no amount of antitoxin will save the animals

Given after 10 minutes	5 units antitoxin
Given after 20 minutes	200 units antitoxin
Given after 30 minutes	2 000 units antitoxin
Given after 45 minutes	4 000 units antitoxin
Given after 60 minutes	5,000 units antitoxin
Given after 90 minutes	No amount

Observations of a very similar nature were made by Strauss and Hendee (1959) on HeLa cells growing in tissue culture After addition of toxin to the culture antitoxin is capable of reversing or preventing its action only if added within 30 to 60 minutes

The immediate fate of injected toxin which soon renders it unavailable for neutralization by antitoxin is not clearly understood Whatever the explanation the fact itself supplies the guiding principle which must be applied in antitoxin therapy *Treatment must be prompt and adequate*

The physician seeing a patient whose throat suggests the reasonable probability of diphtheritic infection should send a culture to the laboratory but should administer antitoxin at once The laboratory report will later confirm or refute the diagnosis but far less damage will be done by the administration of an occasional unnecessary dose of antitoxin than by delay in its use when it is required The following tabulation of fatality according to the day of the disease on which antitoxin was administered is quoted by Russell (1943)

It is important that no chemotherapy be administered before taking the culture Failure to observe this precaution may lead to false negative results or prolonged delay in growth of the organisms

No definite rules can be laid down for the amount of antitoxin required for adequate therapy The actual amount of toxin which has gained entrance to the tissues on the 1st or the 2nd day of the disease is frequently less than a human lethal dose and probably could be expressed in terms of a relatively few guinea pig minimal lethal doses Were this not so the mortality in untreated diph

to add to the immunization program two or more recall or booster injections in later years in order to avoid future difficulty

The importance of recall injections of tox oid was clearly demonstrated during World War II Prior to the war mass immunization against diphtheria had not been introduced into Germany and the carrier rate remained high In Holland on the other hand artificial immunization of infants had been practiced on a large scale for a number of years Within a year following the German occupation the incidence of diphtheria among the Dutch population as a whole rose 80 fold despite the fact that the diphtheria rate in children under 4 years of age continued to decline A similar increased age incidence of diph theria has occurred in all other countries where mass immunization of children has been introduced

It is evident from the above discussion that within a population in which diphtheria is prevalent it is incorrect to think of the ex istence of two simple groups the susceptible and the immune since the one merges into the other through a series of gradations Those with no previous contact will consti tute one fairly sharp group of high suscepti bility whose blood on examination would show no antitoxin Exactly the same labora tory findings would be manifested by others whose previous contacts with the diphtheria though definite were minimal and by others with more extensive contact who because of lapse of time or personal idiosyncrasy had either lost or failed to produce antitoxin The majority of individuals would possess measurable amounts of antitoxin i e more than 0 001 unit per ml and extending up ward possibly to many units especially if artificial immunization had been employed Clearly then the result of exposure of an individual to infection within such a group would depend on a balance among several factors including at least the virulence of the organism the degree of exposure (size of dose) and most important the immune status of the individual

During recent years outbreaks of diph theria have occurred in Denmark and Great Britain among immunized populations The studies of Ipsen (1946) and of Edward and Allison (1951) demonstrated that on the 1st or the 2nd day of disease a number of cases showed serum antitoxin levels well above 0 01 to 0 03 units per ml This level has generally been assumed to give complete protection from the toxic manifestations of the infection The disease in immunized per sons is usually mild and differs clinically from diphtheria in the completely susceptible in dividual in that there is less edema associated with the local lesion other effects attributable to toxin such as myocarditis and late pa ralysis do not occur Serum taken several weeks after recovery showed in almost every instance a striking increase in antitoxin titer Edward and Allison reported that nontoxi genic strains of *C diphtheriae* are capable of causing a similar disease but that in this case no rise in serum antitoxin level occurs Similar observations have been made in this country by Frobisher *et al* (1947)

The Schick Test

In 1913 Schick described a test based on the fact that when a minute amount of diph theria toxin is introduced intradermally it exerts a local destructive or necrotic effect on the cells of the skin and the underlying tissue If the blood passing through the tissue contains antitoxin it will neutralize the toxin and no injury will occur otherwise a visible reaction develops over a period of days the extent and the severity of which parallel the amount of toxin injected Some individuals (usually immune) may show delayed inflam matory reactions to diphtheria toxin itself to other materials present in toxic filtrates or to peptones used to stabilize the diluted toxin Because these allergic reactions may be con fused with reactions due to the primary tox icity of the toxic protein it is customary to include a control in carrying out the Schick test Until recently this control consisted of crude toxin heated to 65 C for 15 *minutes* a procedure said to destroy the toxic com ponent without affecting the immunologic re activity of other proteins concerned in pro ducing the allergic reaction Since it has been shown that even highly purified toxin is capable of eliciting allergic reactions in cer tain immune individuals (Pappenheimer 1958) toxoid would seem to be more suit able for use as a control than heated toxin The following Schick test materials have

It is clear that circulating antitoxin does not prevent the establishment of the carrier state While avirulent *nontoxinogenic* bacilli have been isolated from the throats of susceptible (Schick positive) persons, the serum of healthy carriers of *toxinogenic* strains has been shown invariably to contain an appreciable titer of antitoxin During the 1920's the carrier rate for diphtheria bacilli in a large city such as London was estimated to be from 2 to 5 per cent and in institutions the rate was often found to be even higher (Russell 1943) It was predicted that with the advent of widespread immunization the carrier rate would rise still further Actually the reverse occurred and the almost universal immunization of urban children with toxoid has been accompanied by almost complete disappearance of the diphtheria bacillus and of the carrier state

Immunity and Epidemiology

In its basic features immunity to diphtheria is probably the simplest concept in the whole field of the study of specific resistance to disease Unfortunately in certain details of theory as well as of application difficulties are encountered

Before World War I and before the present practice of universal immunization of children with toxoid became established surveys showed that a high proportion of the population especially in the larger cities had acquired an immunity to diphtheria by natural means Their blood serum contained readily demonstrable circulating antitoxin Indeed, Schick's first survey of Viennese children in 1913 showed that 93 per cent of them were immune at birth He found that the proportion of children with sufficient circulating antitoxin to give a negative skin test with toxin fell to a low of about 37 per cent between the ages of 2 and 5 and then rose again to over 50 per cent of all children between 5 and 15 How was this natural immunity acquired? It has already been mentioned that the carrier rate among urban populations was high in cities where diphtheria was endemic Therefore the chance that a given individual might avoid contact with a toxinogenic diphtheria bacillus for any prolonged period *must have been correspondingly* slight It is probable that *brief en*counters with virulent organisms by children who still possessed some antitoxin derived from the mother by placental transfer were common and provided a primary antigenic stimulus Even after the mother's antitoxin had disappeared, such 'conditioned' children would show a secondary type of antitoxin response following a later infection They might suffer a relatively mild form of the disease or even an inapparent infection that would be followed by a measurable antitoxin response It is quite possible too that completely susceptible individuals may recover from diphtheria following infection with a small dose of a diphtherial strain of relatively low virulence Thus, before the advent of active immunization diphtheria was primarily a disease of young children and there was a fairly effective level of immunity among older children and the adult population which in crowded areas was maintained by relatively frequent encounters with toxinogenic diphtheria bacilli through exposures to cases of the disease and to healthy carriers

With the introduction of universal artificial immunization the situation changed A primary immunization of infants with 2 or 3 injections of toxoid will quickly induce the formation of a protective level of antitoxin This antitoxin will persist for a variable period depending on factors in the child which cannot yet be defined Duration of the immunity may be only a few months but in most instances it will be a few years In the absence of further artificial stimulus to immunity natural factors again become operative and the immunity may be expected to continue if diphtheria bacilli are abundantly present in the environment However *when the number of cases of diphtheria in* the community becomes sharply reduced through immunization the dissemination of toxinogenic organisms from case to contacts will cease There will be a substantial reduction in the prevalence of diphtheria bacilli Today in many large communities the organisms seem to have disappeared entirely Hence, the more successful the campaign to stamp out diphtheria the more certain it becomes that a susceptible adult population will develop if one depends on immunization *only during infancy* Clearly it is imperative

of children against diphtheria with highly purified toxoid has now become almost universal practice in this country The purified toxoid is usually injected as an alum precipitate or adsorbed on aluminum phosphate gel as adjuvant Purified toxoid combined with tetanus toxoid and pertussis vaccine is commonly administered to infants and young children

Toxoid is prepared by treating a sterile toxic filtrate of the diphtheria bacillus with 0 3 to 0 5 per cent formalin The formalinized toxin is incubated at 37 C and maintained at slightly alkaline pH (about pH 8) until all traces of toxicity have disappeared Then the toxoid protein is purified by alcohol fractionation at low temperatures or by ammonium sulfate fractionation followed by dialysis The final product either alum precipitated or adsorbed on aluminum phosphate gel is diluted so as to contain 10 to 20 Lf per immunizing dose of 0 5 or 1 0 ml and 1 10 000 merthiolate is added as preservative Two doses given 1 month apart are usually adequate for primary immunization followed by a booster dose 1 year later

As a result of the almost universal immunization of children with toxoid diphtheria has become an uncommon disease in almost every civilized country throughout the world Active immunization of infants with diphtheria toxoid combined with tetanus toxoid and pertussis vaccine should be done at the age of a few months

A first booster dose of triple antigen should be administered 1 year after the primary immunization and a second booster dose of diphtheria and tetanus toxoid combined should be given at school age These immunizations should be universal and no Schick testing need be done They should result in a reasonably immune population up to adolescence In older children and adults the situation becomes more complex because hypersensitivity reactions to diphtheria toxoid and/or other diphtherial proteins occur in an increasing proportion of the population The frequency of undesirable reactions to diphtheria toxoid increases with age and is greatest in those communities where diphtheria is prevalent and the carrier rate is high While reactions to toxoid among older children and adults are probably never fatal they may be sufficiently intense to prevent any wholesale immunization of the adult population The reactions are usually of the delayed tuberculin type and may vary in severity from local tenderness and swelling at the injection site to severe generalized illness with fever and complete incapacitation lasting for several days (Pappenheimer *et al* 1950) Reactions of the immediate anaphylactic type are extremely rare although 1 or 2 such cases have been reported in the literature (Kuhns and Pappenheimer 1952)

It is probably best to handle immunization of adults with diphtheria toxoid on an individual basis wherever possible and to carry out preliminary screening by means of the Schick test Individuals reacting to the Schick control toxoid should not be immunized further Experience has shown that such individuals respond to the test itself with a rapid and relatively high antitoxin response In large adult groups such as in military personnel where Schick testing is not feasible the current practice in this country and in Canada is to immunize with tetanus toxoid containing a small amount of diphtheria toxoid (0 5 to 1 Lf per immunizing dose) The small amount of diphtheria toxoid is insufficient to produce severe reactions except in a very few highly sensitive individuals but is sufficient to induce antitoxin production in all but a minor proportion of the population

Diphtheria Antitoxin

Treatment of diphtheria with antitoxin is still the only specific therapy available Antitoxin is generally produced by immunization of horses with toxoid incorporated in adjuvant Once a horse has developed a sufficiently high antitoxin titer (generally 1 000 units or more per ml serum) bleedings are taken and the antitoxic plasma or serum is fractionated with ammonium sulfate and by dialysis to remove serum albumin and water insoluble globulins Horse antitoxin is generally found in the fast moving gamma 1 (or T fraction) of the water soluble or pseudo globulin fraction Further purification and at least partial despeciation is achieved by digestion with pepsin (Pope 1939) The peptic digestion not only causes a decrease in species specificity (Levine *et al* 1952) but also causes a splitting in half of the antitoxin molecule (Peterman and Pappenheimer 1941)

been used in recent years by the Massachusetts Department of Health

1 Highly purified diphtheria toxin so diluted in buffered human serum albumin that 1/50 M L D is contained in 0 1 ml of the solution

2 Highly purified diphtheria toxoid diluted in the same diluent so as to contain 0 01 Lf toxoid in 0 1 ml

By using purified materials in the Schick test the number of persons showing allergic reactions is reduced and restricted almost entirely to the immune faction of the population Because both toxin and toxoid in dilute solution are highly sensitive to surface denaturation it is necessary to add a stabilizing agent to the diluent Human serum albumin is now used for this purpose since the commercial peptones employed in the past as stabilizing agents not infrequently gave rise to severe local and even general allergic reactions in certain sensitive individuals

The test is carried out by a careful intradermal injection of exactly 0 1 ml Schick toxin into the flexor surface of the forearm A similar injection of the toxoid control is made *in the opposite forearm The injected* areas should be inspected at 24 or 48 hours and again between the 4th and the 7th days The following types of reaction can be distinguished

Positive At the site of toxin injection an *area of redness begins to appear* at about 24 hours and becomes progressively more pronounced until it reaches a maximum in about a week At this time it covers an area up to 3 cm or somewhat more in diameter and may show moderate swelling and slight tenderness There is usually a smaller more deeply colored central area 1 0 to 1 5 cm in diameter dark red in color which a few days later turns brown and eventually desquamates sometimes leaving a slightly pigmented surface which may persist for some time The control arm remains completely negative throughout Such a positive test indicates very little or no circulating antitoxin and probable susceptibility to diphtheria

Negative Both arms remain without reaction of any sort Antitoxin is present in reasonable amount sufficient to supply immunity to an ordinary exposure to diphtheria Observations indicate that this level is between 1/30 and 1/100 of a unit per ml

Pseudoreaction Inflammatory reactions appear within 12 to 18 hours at the sites of injection of both Schick toxin and control The reactions reach a maximum within 48 hours (occasionally as late as 72 hours) and then fade in contrast with a true positive Schick reaction which persists for many days Individuals who show pseudoreactions are almost invariably immune to diphtheria *but are hypersensitive to toxin (and toxoid)* or other associated substances present in the solution Almost without exception pseudoreactors show a booster type antitoxin response due to the antigenic stimulation of the Schick test itself

Combined Reaction Commencing like the pseudoreaction delayed reactions develop on both arms After the allergic inflammation has subsided the reaction at the toxin site persists as a positive reaction True combined reactions are seldom observed when purified Schick materials are used in the test They indicate a delayed sensitivity to either toxin or protein impurities present in the preparations and that circulating antitoxin is either absent or present in very low titer *Such individuals almost invariably* respond to the Schick test by antitoxin formation and prove to be negative or pseudoreactors on retest Combined reactions are observed more frequently when commercial peptones are present in the diluent and when crude culture filtrates are used *to prepare the test* materials

The pseudoreactions observed in the Schick test are of the delayed tuberculin type of inflammatory reaction and are probably the result of previous infections usually inapparent with the diphtheria *bacillus* Delayed reactions to Schick test materials are seldom observed in young children their frequency increases with age and is highest among populations that have not been actively immunized and where diphtheria is prevalent

Artificial Immunization

When diphtheria toxin is treated with dilute formalin under suitable conditions its toxicity is lost The detoxified protein is called *toxoid* Toxoid retains all of the immunologic specificity of toxin its injection into animals and man gives rise to the formation of diphtheria antitoxin Immunization

other than *C diphtheriae* itself have rarely been implicated in human disease a number of them may cause disease in animals Of the latter *C ovis* is perhaps the most interesting This strain has been shown to cause severe suppurative lesions in sheep horses and cattle a disease known as pseudotuberculosis Morphologically *C ovis* is a pleomorphic bacillus with metachromatic staining resembling the diphtheria bacillus Colonies are frequently yellow in color Carne (1941) has shown that *C ovis* produces a potent toxin that is antigenically distinct from diphtheria toxin

For the bacteriologist concerned primarily with human disease corynebacteria other than *C diphtheriae* are important chiefly to the extent that they may confuse the diagnosis of diphtheria

C Hofmannii

This organism is often found in the throat either healthy or diseased where it lives as a harmless saprophyte It grows readily on the usual laboratory media including Loeffler s medium and tellurite agar Because of the frequency with which it occurs it is the most troublesome of the diphtheroids in connection with the diagnosis of diphtheria As seen in stained smears the Hofmann bacillus is usually somewhat shorter and more plump than *C diphtheriae* and exhibits a tendency to bipolar staining without the appearance of metachromatic granules The trained observer usually recognizes it without difficulty but assurances comes only with extensive experience On the tellurite plate colonies of *C hofmannii* may resemble closely those of *mitis* strains of the diphtheria bacillus Here also experience aids in differentiation the saprophyte often forming a colony with a rather wide grayish or white margin and a dark central portion whereas the pathogen tends to produce a more uniformly dark or black colony Since there are known to be various serologic types of Hofmann as well as atypical varieties of the diphtheria bacillus it follows that complete certainty must depend on the isolation of the strain and examination of its fermentative properties and its toxigenicity *C hofmannii* fails to ferment glucose and of course lacks the ability to form toxin.

C xerosis

Isolated originally from cases of xerosis conjunctivae this organism was shortly found to be present in many normal conjunctivae and probably is without pathologic significance It is occasionally present in the throat though much less commonly than the Hofmann bacillus Morphologically it is probably indistinguishable from *C diphtheriae* Its growth on artificial media tends to be less vigorous than that of the diphtheria bacillus and the colonies on tellurite plates are somewhat smaller than those of either the *mitis* or the *gravis* strains but larger than those of *intermedius* diphtheria Here again in case of doubt one must resort to isolation fermentation and virulence tests

For a description of other varieties of corynebacteria from both human and animal sources the reader should consult one of the larger handbooks of medical and veterinary bacteriology

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TABLE 4

UNIT OF ACTIVITY	APPROXIMATE MICROGRAMS SPECIFIC PROTEIN PER UNIT
1 unit antitoxin (horse)	10
1 unit antitoxin (horse pepsin treated)	6
1 unit antitoxin (human)	15
1 Lf toxin or toxoid	2 5
1 Minimum lethal dose (M L D) toxin	≦ 0 05
1 Schick test dose toxin	≦ 0 001

ASSAY OF DIPHTHERIA TOXIN TOXOID AND ANTITOXIN

Precise methods for the assay of diphtheria toxin and antitoxin were worked out initially by Paul Ehrlich They furnished a basis for the development of general immunochemical methods for assay of biologically active antigens and their corresponding antibodies in terms of relative combining units Within certain limits and with certain qualifications (Maaloe and Jerne 1952) the *specific activities of both pure diphtheria* toxin and antitoxin have been determined so that relative units can now be translated directly into absolute quantities

Ehrlich early recognized the advantage of using antitoxin rather than toxin as a reference standard because of the former's greater stability By assigning arbitrary units to his reference standard Ehrlich was able to measure the unit potency of other antitoxins by determining their capacity to neutralize a given amount of toxin relative to the standard In 1922 an international standard diphtheria antitoxin was set up and has been maintained at the State Serum Institute in Copenhagen Denmark which periodically distributes samples to control laboratories throughout the world for checking their own standards

A few of the methods for estimating the potency of an unknown antitoxin are outlined briefly below By using antitoxins of known unitage the same methods can be modified for determining the specific combining power of diphtheria toxin preparations

1 By definition one unit of antitoxin when mixed with a quantity of diphtheria toxin called an L + dose (L = limes or threshold) and injected subcutaneously into a 250 Gm guinea pig will cause death of the animal within 4 to 5 days The L + dose of a given toxin is determined against a standard antitoxin and then is mixed with dilutions of the unknown serum and injected into guinea pigs

2 The ability of the unknown serum to neutralize a given toxic preparation may be compared with that of an antitoxin of known potency by titration in the skin of rabbits or guinea pigs (see Fraser, 1931 for details) Provided that one is dealing with antitoxins of high avidity (Maaloe and Jerne 1952) the antitoxin content of an unknown serum *can be estimated with an accuracy* of ± 10 per cent or better by rabbit intracutaneous test The method is one of the most sensitive known for determining antibody and as little as 0 001 unit (Ca 0 01 mcg antitoxin protein) can be detected

3 The Ramon flocculation reaction provides an in vitro method for determining the potency of an unknown serum provided that *sufficient flocculating antitoxin is present* This method is essentially an optimal proportions precipitin test It has been found that when increasing amounts of antitoxin are added to a constant amount of toxin the most rapidly flocculating mixture usually contains an amount of antitoxin just sufficient to combine with and neutralize the toxin When a flocculating antitoxin of known unitage *is employed* the method can also be used to determine the combining power of either toxin or toxoid in Lf units The flocculation reaction although accurate is considerably less sensitive than the in vivo methods By determining the amount of specifically precipitable protein in the Ramon flocculation test and from the specific activities of highly purified toxin and toxoid the amount of specifically active protein contained in the various units can be estimated and are summarized in Table 4

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The corynebacteria are widely distributed in nature and the natural habitat of many strains appears to be the soil While strains

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tions for 24 hours in aqueous alkaline methylene blue solutions Ehrlich in 1882 showed that the tubercle bacilli can be stained with basic dyes in the presence of aniline oil and remain stained after treatment with strong nitric acid which decolorizes other microorganisms The acid fast staining method in common use today was developed by Ziehl in 1882 and subsequently slightly modified by Neelsen More recently it has been found possible to stain with basic fuchsin without heating the specimen by incorporating various surfactants in the staining mixture (Aubert 1950)

In animal lesions tubercle bacilli occur as rod shaped microorganisms On artificial cultivation they vary from coccoid to long filamentous forms depending on the type (bovine human avian etc) the particular strain the age of the culture and environmental conditions The typical rods are straight or somewhat bent with parallel sides and rounded ends in the animal body they vary in length from 1 to 4 microns and from 0 3 to 0 6 micron in diameter No definitive capsule has been demonstrated either in susceptible hosts or in culture media

Ethanol containing 3 per cent hydrochloric acid (acid alcohol) will decolorize all rod forms of other bacteria within a few minutes but pathogenic mycobacteria including the tubercle bacilli will resist this treatment for many hours Young cells are somewhat less acid fast than older cells until autolysis occurs The acid fastness of tubercle bacilli is dependent not only on their chemical constitution but also on the integrity of their cellular structure since physical trauma is sufficient to render them non acid fast and readily stainable by an aniline dye (Yegian and Vanderlinde 1947)

Many nonpathogenic mycobacteria are less acid fast than tubercle bacilli but are nevertheless difficult to decolorize with alcohol alone therefore they are more properly designated as alcohol fast

Mycobacteria cannot be classified as gram positive or gram negative by the Gram staining technic because once they have been stained by basic dyes they cannot be decolorized by alcohol regardless of whether or not they have been treated with iodine

It has been claimed that tubercle bacilli have a life cycle including a very small viable granular form which can pass through ordinary bacteriologic filters These claims are as yet unsubstantiated

Cultivation

The mycobacteria are strictly aerobic They can use for growth a multiplicity of simple carbon compounds as source of energy and ammonia or amino acids as source of nitrogen As yet there is no evidence that they require any of the known vitamins as essential growth factors except biotin under certain circumstances (Schaefer *et al* 1955) and mycobactin for *M paratuberculosis* (Francis *et al* 1953) They need CO_2 for initiation of growth some strains requiring for optimal growth concentrations higher than is available in ordinary air especially on media of acid reaction (Cohn *et al* 1960) The cultivable mycobacteria grow most rapidly between pH 6 0 and 8 0 with an optimum around 6 5 to 6 8 for the pathogenic types They exhibit wide variation in optimal temperature for growth but strains pathogenic for warm blooded animals with two prominent exceptions (see *Mycobacterium ulcerans* and *balnei*) multiply most rapidly between 35 C and 40 C *M avium* being especially tolerant of higher temperatures consistent with the higher body temperature of their natural hosts

Growth of most mycobacteria is slow compared with that of common bacterial species most strikingly so in the case of the human the bovine and the murine types of tubercule bacilli the fastest reported division rate in artificial media corresponds to approximately 18 hours Growth of avian and certain atypical strains of pathogenic tubercle bacilli is more rapid particularly in media containing adequate amounts of fatty acids or fatty acid esters

Three different types of media are in general use for the cultivation of tubercle bacilli Simple synthetic media containing an abundant supply of minerals some simple source of nitrogen such as ammonia glutamate or asparagine and glucose or glycerine are capable of supporting the synthesis of large amounts of bacterial protoplasm

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21

The Mycobacteria

The mycobacteria are classified as a genus (*Mycobacterium*) of the family *Mycobacteriaceae* of the order *Actinomycetales* All mycobacteria which have been intimately associated with disease of man or animals exhibiting intracellular parasitism, are stained with dyes only with difficulty and once stained resist decolorization by strong mineral acids Thus they are strongly acid fast The pathogenic mycobacteria include the tubercle bacilli human type (*Mycobacterium tuberculosis* var *hominis*) bovine type (*M bovis*) and avian type (*M avium*) Hansen s bacilli (*M leprae*) Johne s bacilli (*M paratuberculosis*) *M ulcerans M balnei M kansasii M fortuitum* and Battey and other strains of nonchromogenic and scotochromogenic mycobacteria which are not yet formally classified There are many species of nonpathogenic mycobacteria for example the smegma bacilli (*Mycobacterium smegmatis*) found on man the timothy bacilli (*Mycobacterium phlei*) the butter bacilli (*Mycobacterium butyricum*) and many others found in the soil (for review of taxonomic relationships see Gordon and Smith 1953) The property of acid fastness is also possessed but only to a moderate extent by a number of types of pathogenic nocardia and by certain bacterial spores

The pathogenic mycobacteria cause chronic diseases with lesions of the infectious granuloma type The fully developed lesions of tuberculosis leprosy and Johne s disease have in common certain histologic characteristics collections of epithelioid and giant cells are conspicuous but the type of necrosis called caseation occurs only in tuberculosis

MYCOBACTERIUM TUBERCULOSIS

History

The tubercle bacilli cause such a wide variety of lesions and clinical symptoms in man and animals that before their discovery the pathologist and the clinician were unaware that diseases such as miliary tuberculosis, tuberculous caseous pneumonia tuberculosis of cervical lymph nodes (scrofula) and lupus vulgaris (tuberculosis of the skin) had a common etiology Modern knowledge of tuberculosis began with Laennec who in 1819 described some of the prominent macroscopic aspects of tuberculous lesions and recognized the essential unity of early semitransparent tubercle and of the caseous tuberculous lesions Villemin in 1865, demonstrated that material from the human tuberculous lung could produce tuberculosis in the rabbit later he transmitted tuberculosis from cattle to rabbits

The discovery of tubercle bacilli by Robert Koch in 1882 was preceded by the discovery of the leprosy bacilli by G A Hansen in 1878 These discoveries were made without knowledge of the acid fast tinctorial properties of these microbes staining being done by immersing the prepara

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Three different types of media are in general use for the cultivation of tubercle bacilli Simple synthetic media containing an abundant supply of minerals some simple source of nitrogen such as ammonia glutamate or asparagine and glucose or glycerine are capable of supporting the synthesis of large amounts of bacterial protoplasm

yielding upward of 25 Gm (dry weight) per liter within 3 to 5 weeks of incubation

These results can be obtained only by inoculating such media with billions of cells and providing for intensive aeration for example by violent shaking during incubation On the other hand growth of small inocula of many strains often of single cells can be obtained in a variety of media containing complex organic materials—animal serum egg yolk potato extract charcoal starch etc It is this second type of medium which has been used most extensively for diagnosis and for counting single cells The failure of development of small inocula in the simple synthetic media is due primarily to the presence in the latter of substances which exert an inhibitory effect on growth and exist as impurities in the reagents or on the glassware Traces of toxic lipids and of other surface active agents are probably the most common offenders The growth inhibitory effect of many toxic agents particularly of long chain fatty acids can be neutralized by the addition of adequate amounts of animal serum (Youmans 1944) or of the albumin fraction of serum (Dubos and Davis 1946)

Indeed far from being inhibitory certain fatty acids (the C14 to C18 acids) stimulate the growth of many strains of tubercle bacilli in synthetic media to which serum albumin or other fatty acid binding substances have been added These observations have led to the development of a third better defined type of culture media for pathogenic mycobacteria They consist of synthetic nutrient mixtures to which are added serum albumin and either certain fatty acids usually oleic or a synthetic nontoxic water soluble ester of one of these fatty acids (Dubos and Middlebrook 1947) In the oleic acid–albumin medium as in the classic media most strains of tubercle bacilli characteristically grow in the form of large clumps pellicles or heaped masses due to the hydrophobic character of the bacterial surface The water soluble esters of fatty acids commercially known as the Tweens are capable of wetting the surface of tubercle bacilli and thus allowing them to grow more dispersed in a liquid medium This manner of growth has found a very usef[illegible] in experimental work whenever dispersed growth of these organisms is desired

For primary isolation of tubercle bacilli from pathologic materials an oleic acid–albumin medium has been shown to be as effective as the classic complex organic media containing egg yolk, etc (Middlebrook and Cohn 1958) Microscopic examination of the surface of transparent agar oleic acid–albumin medium permits earlier detection of mycobacterial colonies than is possible with the classic complex media and certain other tests such as the catalase test can be applied directly on the colonies

None of the known biochemical activities of tubercle bacilli is sufficiently characteristic to be useful in defining the group All degrees of variation exist between the various types The saprophytic strains of mycobacteria grow well on most media and their growth is in general more rapid they can often be distinguished by this character alone Moreover on complex media colonies of most strains of saprophytic mycobacteria manifest more pigmentation in the dark (usually yellow or orange) than the parasitic forms and most important the individual bacilli are less acid fast than are virulent human bovine and avian tubercle bacilli

Resistance to Physical and Chemical Agents

Tubercle bacilli are as susceptible to desiccation heat radiation and physical agents as other nonsporulating bacteria However they are sometimes regarded as possessing unusual resistance to antiseptics and chemotherapeutic agents The fact that many tubercle bacilli survive exposure to acids and alkalies for prolonged periods of time is made use of in the isolation of these organisms from pathologic material However it must be emphasized that many tubercle bacilli die during this treatment and that on the other hand many other bacterial species gram positive cocci in particular can survive and grow unless inhibited by more specific means

It is probable that the hydrophobic character of the cell wall accounts in part for the resistance of tubercle bacilli to certain toxic agents It is of interest that Tween 80 in

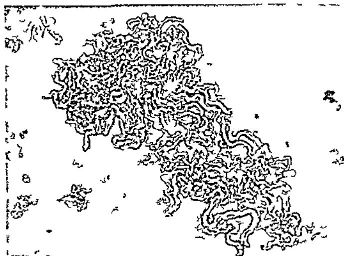


FIG 1 Photomicrograph of microcolony of strain H37Rv (fully pathogenic) showing serpentine cord formation characteristic of virulent human or bovine tubercle bacilli (× 400)

creases their susceptibility to a number of antibacterial agents penicillin for example probably by wetting the bacterial surface (Kirby and Dubos 1947) The resistance of tubercle bacilli to toxic agents has been somewhat exaggerated Nevertheless in practice they are less affected than other species by ordinary antiseptic agents (mineral acids alkali quaternary ammonium compounds) It is possible to add to culture media used in diagnostic work concentrations of penicillin or of malachite green which do not inhibit the growth of pathogenic mycobacteria but prevent the multiplication of gram positive cocci which have resisted preliminary treatment with alkali acid or quaternary ammonium salts (Hirsch 1954)

Tubercle bacilli can survive for long periods of time in the dried state in sputa and excreta Disinfection of such materials can be effected by exposure to various anionic detergents

Variation and Virulence

It has long been recognized that cultures of virulent tubercle bacilli may become attenuated during prolonged repeated subcultivation on artificial media Variation in the gross morphologic appearance of cultures of mammalian tubercle bacilli has also been noted (Oatway and Steenken 1937)

However the unstable and uncontrollable physical and chemical characteristics of the commonly employed solid egg media led to much confusion in description and terminology of colony morphology

Virulent strains of human and eugonic* bovine tubercle bacilli† form microscopic *serpentine cords* of varying thickness and length consisting of strongly acid fast bacilli oriented in parallel with the long axis of the cords (Fig 1) The formation of cords appears to be an important factor in conditioning the ability of virulent cultures to spread on the surface of liquid and solid media On the other hand those eugonic variant strains of mammalian tubercle bacilli which fail to form cords growing in a more or less nonoriented fashion are avirulent (Middlebrook *et al* 1947)

Certain strains of tubercle bacilli possess relatively stable characteristics which are intermediate between those of the fully virulent cord forming strains and the completely avirulent non-cord forming variants Strains of BCG (bacillus of Calmette and Guerin) fall into this group (see Immunization) Furthermore studies (Suter and Dubos 1951 Dubos *et al* 1957) of the relative virulence of colonial variants of 3 different

* See section on characteristics of the different types of *M tuberculosis*

† Nearly all such cultures on primary isolation have uniform high pathogenicity for guinea pigs except cultures from cases of lupus vulgaris (tuberculosis of the skin) and from patients treated with isoniazid which often have diminished pathogenicity

TABLE 1 CHARACTERISTICS OF CULTURABLE MYCOBACTERIA CAUSING DISEASE IN MAN

Species of Mycobacteria	Colony Description: Oleic Acid-Albumin-Agar Medium	Pigmentation: Grown in Dark	Pigmentation: Exposed to Light	Growth Rate (Minimal Inocula): 30 C	37 C	45 C	Neutral Red Test	Niacin Test	Drug Susceptibility or Resistance: Isoniazid 0 2 mcg / ml	Streptomycin 2 mcg / ml	P Aminosalicylic Acid 3 mcg / ml	Catalase	Animal Pathogenicity: Guinea Pig	Chicken	Rabbit	Mouse
M tuberculosis	Dry friable	None	None	0	2–4 wks	0	+	+	S R	S S	S S	+++ 0 to +++	++++ ±	0 0	± 0	++++ ++++
M bovis	Dry friable	None	None	0	2–4 wks	0	+	0	S	S	R	+++	++++	0	++++	++++
*M avium**	Small thin or droplike	None	None	0	3–4 wks	2–3 wks	+	0	R	R	R	++++	0	++++	++++	++++
M ulcerans	Like *M avium*	None	None	3–5 wks	0	0	+	0	R	R	R	+++	0	0	0	0‡
*M balnei**	Smooth butyrous (rarely rough)	None	Yellow	1–2 wks	0	0	0	0	R	R	R	+++++	0	0	0	0‡
*M kansasii** (Group I)†	Smooth butyrous (rarely rough)	None	Yellow	2–3 wks	2–3 wks	0	0	0	R§	R	R	+++++	0	0	0	++++
Unclassified* (Group II)† (Scotochromogens)	Smooth butyrous	Yellow to orange	Yellow to orange	2–3 wks	2–3 wks	0	0	0	R	S or R	R	+++++	0	0	0	0
Unclassified* (Group III)† (Battey strains)	Small thin or droplike	None	None	2–3 wks	3–4 wks	0	0	0	R	R	R	+++	0	0	0	0 or ++++
Unclassified* (Group IV)† Rapid Growers and *M fortuitum*	Rough or smooth	Usually buff	Usually buff	2–4 days	2–4 days	0 or 2–4 days	0	0	R	S or R	R	+++++	0	0	0	0 or ++++

* These species are distinguishable serologically
† According to Timpe and Runyon 1954
‡ Produces lesions in foot pads
§ Sensitive to 1 mcg /ml

BCG strains have indicated that as was to be expected virulence is a function of other as yet unidentified properties of the bacterial cells Cord formation is a necessary but not sufficient condition for virulence of human and bovine types of tubercle bacilli

Strains of tubercle bacilli which produce cords in artificial media are able to bind the dye neutral red in the form of its red salt in alkaline aqueous media on the other hand the non-cord forming variants of tubercle bacilli and nearly all saprophytic strains do not stain red under the same conditions (Dubos and Middlebrook 1948) (see Pathogenesis)

Characteristics of Mycobacteria Causing Disease in Man

In Table 1 are summarized the various characteristics including experimental animal pathogenicity of the cultivable mycobacteria which can cause disease in man Infection tests in the rabbit and the guinea pig will usually suffice to differentiate between human bovine and avian strains freshly isolated from diseased hosts The human type has high pathogenicity for the guinea pig and low pathogenicity for the rabbit The avian type has high pathogenicity for the rabbit in addition to pathogenicity for fowl usually producing in the rabbit the so-called Yersin type* of disease and has very low pathogenicity for the guinea pig A fully virulent bovine culture has high pathogenicity for both the guinea pig and the rabbit † Strains of bovine tubercle bacilli which are somewhat attenuated may have high pathogenicity for the guinea pig but low pathogenicity for the rabbit and therefore can be confused with human strains

Mammalian strains of tubercle bacilli (human and bovine) can usually be distinguished on primary isolation by cultural characteristics On primary isolation bovine strains grow poorly on the usual solid egg media and are inhibited by concentrations of glycerine above 0 75 per cent such cultures are called dysgonic Human type strains on the other hand usually grow more luxuriantly on the solid egg media and are more resistant to glycerine which indeed favors their growth at least as far as the potential yield is concerned they are termed eugonic Dysgonic cultures produce small smooth flat or hemispherical colonies with even entire edges on egg media eugonic cultures grow in the form of rougher usually more spreading colonies with thin uneven edges on egg media However some dysgonic cultures have human type pathogenicity and generally such distinctions between bovine and human cultures are not reliable for conclusive type determination After repeated transfers on artificial media many dysgonic bovine cultures assume the eugonic characteristics of human cultures without change in pathogenicity or virulence

Avian type bacilli on primary isolation from diseased fowl or swine grow readily in the form of smooth hemispherical colonies on egg media They tend to grow somewhat more dispersed than do mammalian strains in the depth of liquid media Dissociation of avian cultures to variants similar in colony morphology to those of human strains has been described These changes may or may not be associated with a change in virulence but they never are accompanied by a change in specific host pathogenicity that is such cultures never become pathogenic for the guinea pig (Feldman 1938)

M avium is distinguishable from the mammalian strains by serologic methods (Furth 1926) The former possesses an antigen or antigens absent in the latter and in tuberculins prepared from yellow bacilli *M fortuitum* *M ulcerans* and *M balnei* Avian strain tuberculin is also distinguishable from mammalian strain tuberculin by quantitative skin tests Human and bovine strain tuberculins are so similar as to be considered identical in skin tests although differences between protein fractions of these strains can be recognized by serologic methods (Schaefer 1947)

Since 1948 an increasing number of distinctly different mycobacteria have been un

* The intravenous injection of large numbers of bovine or avian tubercle bacilli into the rabbit causes a rapidly fatal septicemia with the formation of tubercles of microscopic size

† R Koch maintained that there is only one type of mammalian *M tuberculosis* The credit for distinguishing the human and the bovine types is due to Theobald Smith 1898

equivocally identified with granulomatous lesions in man (principally pulmonary cu taneous and lymphatic) *The characteristics of M ulcerans* and *M balnei* are described in Table 1 The so called yellow bacilli now identified as *M kansasii* constitute a well-defined group of strongly acid fast bacilli larger than human or bovine bacilli and causing pulmonary lesions in man They form either smooth or rough colonies on oleic acid–albumin solid agar media and are characteristically photochromogenic syn thesizing a bright yellow pigment only after exposure to visible light (Buhler and Pollack 1953)

The other cultivatable mycobacteria *which can cause disease in man have been* tentatively classified (Timpe and Runyon, 1954) into scotochromogens (Group II) Battey * strains (Group III) and the so called rapid growers (Group IV) includ ing *M fortuitum* These strains of non chromogenic and variably scotochromogenic mycobacteria are all easily cultivated in vitro at 37° C They are difficult to distin guish from each other except by growth rate and certain biochemical reactions but they appear to be serologically classifiable into distinct groups (Schaefer 1963) The scotochromogens have been associated with disease of cervical lymph nodes in children while the Battey strains are most commonly agents of pulmonary disease (Runyon 1959) The rapid growers (*M fortuitum* etc) are rarely implicated in disease of man and are often distinguished by their viru lence for mice infected with massive num bers of these organisms by the intravenous route (Gordon and Smith 1955 Wells *et al* 1955 Kushner *et al* 1957)

Voles suffering from natural tuberculosis yield strains of acid fast bacilli which differ sufficiently from the other types to be re- ferred to as the murine type (*M microti*) This type has low pathogenicity for the guinea pig and the rabbit *but is pathogenic* for the mouse (Wells 1946)

Murine strains grow more slowly on egg media than do other tubercle bacilli their growth is inhibited by glycerine They are *not distinguishable serologically* from the human and the bovine strains (Wells 1946) *M microti* is not pathogenic for man and has been employed for purposes of vaccination (see Immunization)

Bacteriophages active against saprophytic and pathogenic mycobacteria have been found (Gardner and Weiser 1947, Froman *et al* 1954, Sellers *et al* 1957 Redmond 1963) Phages have been suggested for pos sible use in classification of mycobacteria (Froman, 1953)

* Named after Battey State Hospital Rome Ga where the first series of patients with pulmonary disease associated *with these organisms* was ob- served

M kansasii constitutes Group I of Timpe and Runyon

Pathogenic Properties of the Different Mycobacteria for Man

The human and the bovine types are the principal agents of tuberculosis in man The human type is usually found in cases of pul monary tuberculosis In countries where bo vine tuberculosis is not uncommon the bovine type is often the agent of bone and joint tuberculosis and tuberculous cervical lymphadenitis which occur most often in children This apparent difference in organ specificity of the two principal mammalian types is due in reality to the fact that the route of primary infection determines in large measure the predominant organ pat tern of tuberculous disease Infection by in halation tends to produce pulmonary dis ease whereas primary infection by the gastrointestinal tract (as from ingestion of unpasteurized milk) tends to result in the *extrapulmonary patterns of disease* In rural areas where cattle tuberculosis is widespread (few in the United States) the bovine type is not an uncommon agent of pulmonary tuberculosis The human type is not more *pathogenic for man than the bovine type*

Although there have been described a few well authenticated cases of progressive tu berculosis in man due to the avian type of tubercle bacilli (Feldman 1938 Bradbury and Young *1946*) *they are very rare The* other less commonly encountered patho genic mycobacteria appear to follow the general pattern already established for *M tuberculosis* and *M bovis* However, their *epidemiology is obscure and factors related* to depression of host resistance locally in the lungs, or generally appear to play a

most prominent role in disease associated with these organisms (Corpe *et al* 1963)

Chemical Constituents of Mycobacteria

The relationships between chemical constituents and pathogenicity or virulence of mycobacteria are poorly understood The chemical constituents of these organisms can vary qualitatively and quantitatively depending on the method of cultivation Nevertheless the following information is available as to the chemical constituents of mycobacteria and their relationship to the immunology and the pathology of tuberculosis (Long 1958)

The proteins synthesized by tubercle bacilli are of special significance because they elicit the tuberculin reaction It appears that all of the protein fractions of any particular type of tubercle bacilli can evoke the tuberculin reaction in an animal such as the guinea pig sensitized with the homologous type No information is available as to whether one or several specific polypeptide or protein configurations are responsible for tuberculin activity Certain protein fractions isolated from tubercle bacilli (and other mycobacteria) can induce the formation of precipitins agglutinins and complement fixing antibodies as well as anaphylactic sensitization However injection of these proteins into normal animals does not induce the delayed type of sensitivity responsible for the tuberculin reaction The proteins of human and bovine types cannot be distinguished from one another by skin sensitization The proteins of avian tubercle bacilli Johne s bacilli leprosy bacilli and saprophytic mycobacteria can be distinguished readily from those of mammalian tubercle bacilli by serologic means and by quantitative skin tests in hypersensitive individuals but there are cross reactions throughout the genus *Mycobacterium* This cross reactivity does not interfere with the practical usefulness of the tuberculin test in strongly hypersensitive individuals (see Tuberculin) Extensive studies have been made on the separation and the purification of the proteins of mycobacteria (Seibert 1950)

Tubercle bacilli contain serologically active and inactive polysaccharides of high molecular weight including glycogen (Heidelberger and Menzel 1937 Stacey 1955) The mammalian tubercle bacilli appear to contain at least two serologically distinct polysaccharides Tuberculous hosts may become hypersensitive to homologous polysaccharides this sensitization is of the anaphylactic type—distinct from the tuberculin type (Enders 1929) (see Allergy) The roles of the polysaccharides of tubercle bacilli in the immunity and the pathogenesis of tuberculosis have not been established

The mycobacteria have long been known to be very rich in lipids The systematic investigations of Anderson and his associates (Anderson 1939 1940) revealed a great variety of complex lipids branched fatty acids waxes and higher alcohols Many of the lipids are probably bound to proteins and polysaccharides in the bacterial cell some more firmly than others As the lipids are not easily dissolved or digested in the animal tissues they are probably responsible for certain aspects of the cellular reactions to tubercle bacilli (Sabin 1941) A cellular response resembling the tubercle (see Lesions Caused by Tubercle Bacilli) can be produced by crude phosphatide fractions isolated from tubercle bacilli grown in vitro Certain other lipid fractions obtained from tubercle bacilli or saprophytic mycobacteria or from unrelated sources provoke similar reactions However even when these lipids of tubercle bacilli are used relatively large quantities are required to produce tubercle like lesions or caseation necrosis There are qualitative and quantitative differences in the fatty acids of the lipids extractable from different types of mycobacteria (mammalian avian saprophytic) cultivated on the same medium and they vary with changes in the chemical composition of the medium and with the age of the cultures (Cason *et al* 1956 Asselineau 1956)

Recent studies of the wax fractions of mycobacteria have given more precise knowledge of the structure of the high molecular weight alpha branched chain beta hydroxy fatty acids (mycolic acids) which occur free and esterified to glycerol oligo saccharides and polysaccharides in these organisms Unesterified mycolic acids are the only lipids from tubercle bacilli which are

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acid fast per se An antigenic 'lipopolysaccharide, first obtained from tubercle bacilli by Choucroun has been shown to contain in addition to mycolic acids and a serologically active polysaccharide, 3 amino acids, includ ing diaminopimelic acid (Asselineau 1952)

A *toxic glycolipid first extracted from* living virulent tubercle bacilli by petroleum ether (Bloch 1950) has been identified as trehalose 6 6 dimycolate (Noll *et al*, 1956) (see Pathogenesis)

Another lipid sulfolipid was first ex tracted from moist living bacterial cells of a virulent strain of tubercle bacilli with petro leum ether containing a small amount of an aliphatic amine (Middlebrook *et al* 1959) This substance obtained in large yield only from virulent strains of *M tuberculosis* strongly fixes the dye neutral red and ap pears to be responsible for this characteristic of virulent strains of tubercle bacilli (Gan gadharam *et al*, 1963) Its complete struc *ture has not been elucidated but the sulfur* is present in the form of a sulfuric acid ester of trehalose

The probable role of the complex lipids in the staining properties of mycobacteria has not been clearly established but it is known that the cells remain acid fast after treatment with neutral fat solvents and that treatment with 1 per cent hydrochloric acid in ether alcohol solution destroys their acid fastness This acid treatment allows extrac tion of certain firmly bound lipids which are otherwise not extractable from the cells with neutral fat solvents such as alcohol ether or chloroform However there is no doubt that acid fastness is not due merely to free mycolic acids

TUBERCULOSIS

The host parasite relationship is of such a nature in tuberculosis that the immunology and the pathology of the disease cannot be discussed separately During the course of the infection the character of the cellular response of the host changes and the para sites find modified conditions for their mul tiplication and dissemination The relation ship between specific antigenic components of mycobacteria and mechanisms of host protection has not been elucidated In tuber culosis, acquired specific resistance i e, im munity, can be approached as yet only by description of events during the course of infection as seen in its morphologic pathol ogy Therefore the pathologic aspects of *tuberculosis will be emphasized here*

Pathogenesis

Until recently there was no evidence re lating any particular properties of virulent tubercle bacilli to virulence Correlation be tween morphologic patterns of growth and virulence led to the hypothesis that cord formation is the consequence of the accumu lation of lipid material about the bacilli binding them together and surrounding them with one or more substances assumed to play a part in pathogenicity (Middlebrook, 1950a) Indeed it was shown subsequently that living or killed cells of virulent tubercle bacilli can inhibit the migration of leuko *cytes of normal susceptible hosts in vitro* as well as in vivo (Allgower and Bloch 1949, Martin *et al*, 1950) Virulent tubercle ba cilli and certain of their culture filtrates also exert a primary toxic effect on phagocytic leukocytes, resulting in leukocytic degenera tion after various periods of time (Suter, 1952, Fong *et al* 1957) Under the same experimental conditions, non-cord forming avirulent variants of these organisms and some cord forming attenuated strains ap pear to be much less active in these respects Finally Husseini and Elberg (1952) have observed that phthienoic an alpha beta un saturated trimethylated C_{27} fatty acid from virulent tubercle bacilli is very active in inhibiting the migration of leukocytes in vitro

Extraction of living cultures of virulent tubercle bacilli with petrol ether a solvent which disperses virulent bacilli from their characteristic orientation, yields a complex *mixture of substances which inhibits leuko* cytic migration as do the whole bacilli (Bloch, 1950) Because this fraction was extracted from the bacilli by a solvent which disrupts the characteristic cords of virulent strains it has been called cord factor Subsequent fractionation of the petroleum

ether extracts has yielded the toxic lipid trehalose 6 6 -dimycolate * The possible correlation of these findings with those of Choucroun and Asselineau and the recent isolation of a toxic lipid material from tubercle bacilli by Spitznagel and Dubos (1955) is not yet clear (Bloch *et al* 1957)

It seems likely that some substance or substances accounting for the characteristic serpentine cord formation and neutral red binding capacity of cultures of virulent tubercle bacilli play a significant part in the pathogenesis of tuberculosis It is tempting to postulate that the recently isolated sulfolipid is this substance because it appears to have the appropriate properties but its chemical nature and the mechanism by which it may operate as a virulence factor still remain to be clarified It is significant that none of the toxic lipid fractions mentioned above binds neutral red in salt form

In addition to the subtle primary effect of virulent tubercle bacilli on the normal physiologic activity of phagocytes there develops during infection an allergic hypersensitivity to tuberculoproteins—and possibly to other components of the bacteria The early appearance of hypersensitivity modifies the host parasite relationship in very complex fashion and is an inseparable part of natural tuberculous infection The clinical symptoms of general toxicity in this disease are probably in large part a consequence of acquired systemic hypersensitivity to metabolic products of the parasite

In many of the typical acute infectious diseases due to bacteria the pathogenic agents multiply uninterruptedly until the host recovers or dies as in uncomplicated lobar pneumonia or diphtheria In tuberculosis however the infectious agent at first multiplies unopposed and later due to the development of some degree of acquired resistance multiplication becomes restricted but rarely are all the living parasites completely eliminated from the host Indeed a few viable tubercle bacilli may survive in the primary lesions and even after many years may originate newly progressive disease Thus there is usually established a delicate equilibrium between the host and the parasite which can shift in favor of the host or the parasite with a recurrent cycle of progressive and quiescent disease It is characteristic for tuberculosis that even in the same organ healing and progressing lesions may coexist In other words it appears that in every focus of infection a process goes on influenced in part by ystemic and in part by local factors

Many studies have shown that the administration of cortisone promotes tuberculous infection in certain experimental animals In man the usual therapeutic doses of ACTH or cortisone decrease the signs and the symptoms of clinical disease at least temporarily (LeMaistre *et al* 1951 Cocchi 1956) On the other hand these hormones can enhance the infectious process in man as well as in rabbits (Lurie 1955) Cortisone depresses but does not completely abolish hypersensitivity to tuberculin

Today tuberculous infection in man takes place almost exclusively by means of inhalation of a single tubercle bacillus or at most a few bacilli in a droplet nucleus of sputum which is deposited deep in the air ways of the respiratory tract beyond the origin of the ciliated epithelium (Jensen 1960) It is seldom that more than one primary focus results The tubercle bacillus so deposited is promptly taken up by a fixed mononuclear phagocyte in the alveolar duct where it starts its intracellular multiplication

By histologic and bacteriologic examination of the lungs the mediastinal lymph nodes and the spleens of guinea pigs at different intervals of time after aerogenic infection with living virulent tubercle bacilli one can observe in this experimental model what probably happens under natural conditions in human beings This is possible at least during the first few weeks after implantation In the previously uninfected individual a long time elapses before a local process general processes or symptoms arise The tubercle bacilli multiply in the original host phagocyte which is immobi

* Repeated injection into mice of very small amounts of paraffin oil solution of this substance brings about hemorrhagic pulmonary lesions and death after a period of days The same fraction is much less toxic for mice when injected as a single dose even in much larger amounts

lized near the site of implantation When the number of intracellular bacteria becomes excessive the phagocyte bursts and new phagocytes beginning to accumulate in the lesion share the burden of the logarithmically increasing numbers of organisms In the guinea pig even with the most virulent strains no lesions are visible to the unaided eye earlier than the 14th day The tubercle bacilli in the numbers formed before host responsiveness develops appear to be relatively nontoxic for the previously uninfected animal However sufficient antigen is released to sensitize the host to tuberculoprotein within 3 to 4 weeks With the development of host responsiveness an acute inflammatory process followed by central necrosis appears at the site of the primary focus which now contains very large numbers of bacilli approximately 1 000 000 bacilli per lesion at this time in the guinea pig

During the preallergic phase a few of the bacilli escape or are carried by phagocytes from the primary focus through lymphatic channels to the regional lymph nodes where there are also large numbers of bacilli by the time host responsiveness develops Somewhat later still at about the time of the development of host responsiveness some of the bacterial cells have found their way probably by a hematogenous route to the spleen Thus begins the whole process of tuberculous infection in disease In the guinea pig the average incubation period *before the development of hypersensitivity is* about 21 days in man about 40 days The subsequent fate of the tubercle bacilli and the host lesions is different in different species and in different individuals within the species apparently depending on the rate of host responsiveness as emphasized by Lurie in his studies of genetically different races of inbred rabbits (Lurie 1955)

It is important to note that silica (SiO) dust locally decreases host resistance to tuberculosis and other mycobacterioses both in experimental animals and in human beings (Vigliani and Pernis 1963) No satisfactory explanation has been given so far for this phenomenon though it would appear that the silica so injures immune mononuclear phagocytes as to interfere with their ability to inhibit intracellular multiplication of the bacteria

Lesions Caused by Tubercle Bacilli

Human beings respond to tubercle bacilli and their products with two types of lesions in general One type is called exudative the other, productive or proliferative The exudative type resembles lesions caused by pyogenic bacteria at least in some respects in the lungs, there is an exudate in the alveolar spaces and the elements of the normal tissue are included This lesion in the hypersensitive host may be microscopic or it may involve an entire lobe—most often seen when large numbers of bacilli reach an undiseased part of the lung by aspiration from anothe part of the cavity It may heal by resolution that is bacillary multiplication is inhibited most of the bacilli are destroyed and tissue healing may take place with a minimum of scarring It may undergo early necrosis sometimes involving a whole lobe and lead to massive sloughing of necrotic tissue and gross ulceration with cavity formation Or it may develop into the productive type of lesion

The productive type is classified as an infectious granuloma because it resembles both granulation tissue and a tumor Because tuberculosis usually has a chronic character with cyclic periods of activity and quiescence with healing the productive lesion is the one most frequently observed in tuberculosis It presumably develops from the exu*dative type in the following manner (Canetti* 1955) After the diminution in exudate and the appearance of mononuclear phagocytes cells of a different type, epithelioid cells appear and increase in number This cell has a pale cytoplasm and a large elongated nucleus The faintly outlined epithelioid cells are of irregular form and are usually arranged side by side At this time acid fast bacilli can be found in these cells but it is noteworthy that only a few cells contain stainable bacilli After 2 or 3 weeks if the lesion does not spread too rapidly a zone of proliferating fibroblasts mingled with lymphocytes appears at the periphery and in the center of the older lesions giant cells may be found These cells are characterized by their large size being up to a few hun

dred micra in diameter and by their numerous dark staining nuclei situated at the periphery of the cytoplasm they also may contain stainable tubercle bacilli The typical lesion now has 3 zones a central giant cell or zone of giant cells a midzone of epithelioid cells arranged radially and a peripheral zone of fibroblasts lymphocytes monocytes and plasma cells supported by a newly formed reticulum

The cellular response just described is characteristic of the productive type of tuberculous lesion and is defined as the microscopic tubercle In human disease it is seen microscopically as a barely visible grayish translucent nodule it may become larger opaque and yellowish as necrosis occurs in the center of the lesion Such a macroscopic tubercle often consists of a coalesced group of microscopic tubercles Thus the tubercle may grow in size by extension or by fusion with other tubercles and become necrotic The necrotic material has the consistency and the appearance of cheese therefore the process is called caseation necrosis The biochemical factors in the production of this peculiar type of necrosis are not clear but



FIG 2 Polymorphonuclear alveolitis The alveoli are filled with polymorphonuclears more or less modified Occasional macrophages and little or no fibrin This lesion can be distinguished from nontuberculous suppurative pneumonia only by the presence of bacilli (× 140) (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 39 Springer New York)



FIG 3 Bacilli in polymorphonuclear alveolitis Bacilli are more numerous than in any other tuberculous exudate (Ziehl × 800) (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 39 Springer New York)

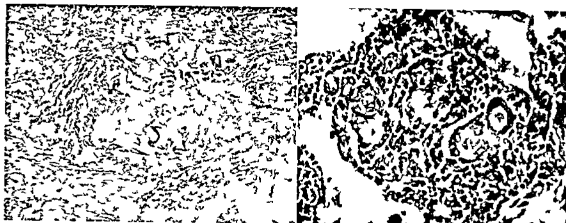


FIG 4 (*Left*) Productive lesion of lung In the center is a giant cell of enormous dimensions Assuming it to be spherical it would contain about 1 000 nuclei No bacilli by Ziehl stain Ordinary Langhans type giant cell beside it (× 80)

FIG 5 (*Right*) Same case Accumulation of numerous giant-cells predominantly of the Langhans type No bacilli by Ziehl stain (× 340) (Canetti G The Tubercle Bacilli in the Pulmonary Lesion of Man p 163 Springer New York)

it is assumed that certain autolytic enzymes of the host which normally effect liquefaction to pus are inhibited A caseous tubercle small or large may break into a bronchiolus or a bronchus and empty its contents this results in cavity formation On the other hand calcium salts may be deposited in the caseous material of the walled-off tubercle The older tubercles may become surrounded by a thick layer of fibrocytes in the form of a capsule and fibrous tissue with vascularization may penetrate and replace the tubercle Calcification and even ossification of healed pulmonary tubercles occur frequently in infancy and childhood

Of importance for the fate of the early exudative lesion are the initial number of tubercle bacilli their rate of multiplication in the particular host and the degree of hypersensitivity of the host A small number of bacilli with little multiplication presumably leads to resolution and disappearance of the lesion sometimes without typical tubercle formation A large initial number of tubercle bacilli or their rapid multiplication (in a host with low resistance) especially in a highly hypersensitive host leads to early and widespread necrosis even at sites where presumably the typical tubercle has no opportunity to form (caseous and gelatinous pneumonia) Slow multiplication of tubercle bacilli continuous or discontinuous results in the proliferative (or productive) type of lesion

The accompanying illustrations (Figs 2 to 5) show the principal characteristics of the two predominant types of tuberculous lesions It must be remembered that both the productive and the exudative types of lesions are usually present in chronic progressive tuberculosis in man they may be anatomically contiguous and lesions intermediate between the two types are frequently evident (Canetti 1955)

SPREAD OF TUBERCLE BACILLI IN THE HOST

Although tubercle bacilli multiply readily in artificial media in the absence of tissue cells they are primarily intracellular in the infected host However they also multiply extracellularly in ulcerated lesions such as pulmonary cavities and an extracellular phase has to be assumed in the progressive spreading infection even in nonulcerated lesions They may spread by contiguity by lymphatic drainage by the bloodstream and by tubular means

Like other microscopic particulate matter tubercle bacilli usually reach the nearest regional lymph node by *lymphatic dissemination* There multiplication may take place and the bacterial cells may pass to other lymph nodes Thus a chain of lymph nodes

may be infected and occasionally passage through the thoracic duct into the blood stream can result in systemic dissemination and miliary tuberculosis When a host is infected for the first time (regardless of whether the portal of entry is pulmonary or intestinal) gross lesions almost always de velop in the draining lymph nodes For this reason the tuberculous foci of the hilar lymph nodes were called by Parrot in 1816 *miroirs des poumons* Tuberculous infection contracted by ingestion results in the in volvement of mesenteric lymph nodes While the infection of the draining lymph nodes occurs with the regularity of biologic law during the first infection lymphatic spread is almost always absent or inconspicuous after reinfection

Hematogenous dissemination of tubercle bacilli occurs most frequently during the progress of first infection tuberculosis and originates as a rule either in a caseous mediastinal lymph node or by the penetra tion of a vein by a growing tubercle The uniform distribution of tubercles in the lung as seen by roentgenograms may allow a diag nosis at this stage Rarely tubercle bacilli may be cultured from the blood Tubercu lous meningitis probably a result of bacte remia, occurs more frequently in children following first infection than in adults who have miliary tuberculosis

The outcome of hematogenous distribu tion depends on organ susceptibility The thyroid gland the pancreas the heart and the voluntary muscles almost never are the sites of miliary blood borne tubercles In parts of the lung compressed because of effusion or pneumothorax there may be fewer and smaller tubercles than elsewhere in the lung However this may be due merely to diminished blood supply

Hematogenous miliary tuberculosis may not be associated with recognizable tubercles in susceptible organs other than the lungs such as the spleen the liver or the kidneys Following hematogenous dissemination the tubercles in the lungs may be exudative or productive or the central part exudative sur rounded by a productive zone In other organs they are as a rule of the productive type Hematogenous spread may occur re peatedly

Tuberculosis of kidney liver spleen bone testes ovaries and other organs is a consequence of blood invasion Occasionally at necropsy of persons dying of causes other than tuberculosis minute tuberculous lesions mostly calcified are found in the kidneys the liver or the spleen

Tubular Dissemination In tubular dis semination the contents of a discharging cavity reach the bronchi with subsequent aspiration into the parenchyma of the lung initiating new pulmonary lesions Tubercu lous laryngitis tonsillitis and enteritis can also result from such a spread Similarly tuberculosis of the kidney may lead to tuber culous cystitis The tubular spread of tuber cle bacilli plays a conspicuous part in adult pulmonary tuberculosis For example a cavity in the upper lobe discharges its con tents into a bronchus Particularly if the discharge material is liquid it may be aspirated into other portions of the same or the opposite lung and there initiate new foci of disease Tuberculous caseous bron chopneumonia is apt to occur following hemoptysis by aspiration of blood containing viable tubercle bacilli

It can be shown in experimental animals that while the lesions of first infection con tinue to progress contiguously bacilli intro duced into normal parts of the same organ often fail to initiate other progressive foci This experimental observation finds its clini cal counterpart in the fact that progressive cavitary disease of the lungs associated with large numbers of tubercle bacilli in the bron chial secretions is not invariably followed by extensive new foci of disease Thus the re sistance of individuals with active tubercu lous infection is often sufficient to cope with many small local infective units of reinfec tion while insufficient to cope with the enor mous bacillary population in the gross estab lished lesion This fact serves as a rational basis for surgical removal of extensive areas of disease in individuals who even after surgery continue to shed some bacilli from smaller lesions in other parts of the lungs

Primary (First Infection) and Postprimary (Reinfection) Types of Tuberculosis

Under the epidemiologic conditions which prevailed until recently most of the first tuberculous infections occurred in childhood

This led to the use of the term childhood *tuberculosis for the primary, first infection* type of disease and adult type for the postprimary or reinfection type of disease In reality the clinicopathologic character of tuberculosis in any individual is determined by the degree of immunity and hypersensitivity of the individual at the time of examination and not necessarily by his age Race and previous immunologic experience with tubercle *bacilli and other antigenically related* mycobacteria as well as many other factors are at least as important as age in determining the degree of resistance and hence the type of disease

Therefore the term primary and 'postprimary are used here and defined as clinicopathologic types of disease without any necessary implications as to the age of the individual or the times of first infection or reinfection

An illuminating analogy of the primary and the postprimary types of tuberculosis can be recognized in experimental animals (see Immunity Koch Phenomenon) When living virulent tubercle bacilli are deposited in the skin or the lungs of a guinea pig the draining lymph nodes are soon involved and progressive disease rapidly ensues If the same guinea pig is infected subsequently in the same way again but at another site on the opposite leg or in another part of the lungs for instance the tubercle bacilli remain entirely or almost entirely localized they may multiply locally but do not or only in small numbers invade the draining lymph nodes This prevention of lymphatic spread is concomitant with the development of some degree of acquired re*sistance to the disease*

In the United States primary tuberculosis is almost always the result of inhalation of the human type of tubercle bacilli in the form of a minute droplet nucleus less than 8 microns in diameter and containing one or very few living bacilli In countries where unpasteurized milk contaminated with bovine type tubercle bacilli is consumed the primary infection arises in the cervical lymph nodes and the intestines in an appreciable number of cases

Primary pulmonary tuberculosis *differs in* many respects from the postprimary type (Ghon 1916 Opie 1917) Whereas the location of the gross lesion of the primary *type may be in* any part of the lung and no more frequently in the apex than elsewhere the prominent postprimary lesion almost always makes its appearance near the apex A significant involvement of a hilar lymph node is present with the primary type but is not associated with the postprimary type of disease Indeed in the primary type an inconspicuous parenchymal lesion is often *associated with massive caseation of the* draining lymph node * whereas in the postprimary type the hilar nodes rarely contain caseous lesions even if whole lobes are diseased The primary type of tuberculosis is an acute disease, healing or progressing in a relatively short time the postprimary type by contrast is more chronic *because it is* associated with a significant—albeit inadequate—degree of resistance The primary type of pulmonary tuberculosis can progress so rapidly that death occurs before cavities develop, while in the postprimary type fibrosis is conspicuous and cavity formation is the rule the disease *usually beginning near* the apex of a lobe and progressing down*ward clearly through tubular routes*

There are histologic differences between the progressive primary and the postprimary types of parenchymal pulmonary lesions The progressive primary type is almost always exudative the postprimary type is predominantly productive with only occasionally *exudative foci*, usually the result of recent tubular spread The primary type often heals by resolution or may undergo caseation and calcification (without ulceration) Healing by resolution is rare in the postprimary type of disease except *under the influence of* effective chemotherapy

The primary type of disease most commonly seen in infants and children may be observed in adults who have escaped earlier infection On the other hand adults acquiring their first infection as indicated by *previous negative tuberculin tests* may manifest the postprimary type of disease passing through a primary phase which remains unrecognized because of the rapid development

* The primary pulmonary complex Ghon complex consists of the primary parenchymal lesion ("Ghon tubercle") and the involved regional hilar lymphatic tissue

of acquired resistance In the United States the rapidly progressive primary type is seen more often in young adult Negroes than in white adults

The postprimary reinfection type of tuberculosis may be caused by—seeded with—tubercle bacilli newly inhaled from without (exogenous reinfection) or by organisms which were present and had survived in the primary lesions (endogenous reinfection) The distinction between endogenous and exogenous reinfection is epidemiologically important but it is often impossible in individual cases to ascertain the origin of the bacilli causing the postprimary disease

The healing of disease of first infection and the appearance of postprimary disease may be separated by an interval of many years The question arises as to how the relative immunity is maintained Do the tubercle bacilli in the healed primary lesion survive and thus maintain the immunologic status of the host? Are they dead but their antigens not completely destroyed and eliminated? Careful studies of healed lesions of primary type disease have shown that in most cases they contain no viable tubercle bacilli on the other hand epidemiologic data show that tuberculin sensitivity once developed is sometimes lasting The explanation in such instances may be that casual contacts with small numbers of tubercle bacilli which multiply briefly are enough to maintain tuberculin hypersensitivity and immunity without causing lesions large enough to be roentgenographically demonstrable This opinion is supported by observations on the persistence and even an increase of tuberculin hypersensitivity in American Indians long after vaccination with BCG (Aronson 1948) It may be added that the primary type of tuberculosis has been observed at autopsy in persons whose lungs had sustained a healed primary complex—healed parenchymal and hilar lymph node lesions (Terplan 1940) It is reasonable to assume that in these persons the complete healing of the first infection lesions was followed years later by complete loss of acquired resistance

Immunity

The term immunity is used here in a restricted sense meaning specific acquired resistance to infection or disease Humoral antibodies appear not to play a role in immunity against tuberculosis The sera of actively immunized animals agglutinate tubercle bacilli in vitro they fix complement in the presence of tubercle bacilli and certain of their antigenic components they form precipitates with protein and carbohydrate fractions of tubercle bacilli and they promote phagocytosis by polymorphonuclear leukocytes and monocytes in vitro Yet such sera even in the presence of complement have no bactericidal or lytic effects on tubercle bacilli

In many pathogenic species of bacteria the virulent variants possess surface antigens which are essential for virulence and antibodies against these confer specific resistance None of the known antigenic substances of tubercle bacilli has been shown to be related to virulence Attempts at passive transfer of acquired resistance to tuberculosis by serum transfer in experimental animals have thus far failed (Raffel 1956)

Infection with tubercle bacilli enhances the host response to antigens not related to tubercle bacilli Antibody formation is nonspecifically increased and sensitization is altered (Dienes and Schoenheit 1927) The addition of paraffin oil to killed tubercle bacilli increases and prolongs antibody formation and sensitization to the antigens of tubercle bacilli The antigenic effect of certain other antigens incorporated in paraffin oil with killed tubercle bacilli is also greatly increased and prolonged (Freund 1956) Thus it is paradoxical that protective humoral antibodies have not been shown to play a role in specific acquired resistance to tuberculosis

In addition to the formation of humoral antibodies the tuberculous host develops another type of immune process namely an altered tissue reactivity to tubercle bacilli and their components called *allergy* It has long been customary to describe the reaction known as the *Koch phenomenon* as illustrating certain aspects of host responsiveness and immunity in tuberculosis

When a guinea pig is injected in the subcutaneous tissue of the thigh with living virulent tubercle bacilli the puncture wound heals within 2 days From 10 to 14 days later a nodule appears at the site of injection It

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losis operates by an as yet undemonstrated cellular mechanism unlike any hitherto known to exist in other infectious diseases and dependent on neither humoral antibodies nor hypersensitivity to tuberculin

First let it be made clear that many students of this problem find the available experimental evidence insufficient to warrant a decision as to which one of the above viewpoints is correct Indeed it seems probable that the many and various phases and forms of tuberculous infection and disease which occur under natural conditions or can be produced experimentally will require a more complex answer to this problem than any one of the above mentioned views indicates For example there is little doubt that the amount of caseation necrosis which can be produced by any specific number of tubercle bacilli is determined in large measure by the degree of hypersensitivity of the host and as has been pointed out above spread of bacilli by tubular routes in the lungs is dependent on previous necrosis sloughing of the necrotic tissue and ultimate cavity formation Thus with respect to this most significant aspect of phthisis hypersensitivity is an unfavorable concomitant of tuberculous disease On the other hand the same focal necrotizing effect of hypersensitiveness probably results in decreased local multiplication of tubercle bacilli by creation of an environment which is physiochemically unfavorable for their growth (Dubos 1950 Sever and Youmans 1957) particularly since tubercle bacilli are strict aerobes

It has not been possible experimentally to confer a high degree of specific acquired resistance with fractions of tubercle bacilli without the concomitance of tuberculin hypersensitivity though some increase in resistance to infection can be conferred without allergy by certain methanol extracts of tubercle bacilli (Negre 1956 Weiss and Dubos 1955) whether or not this increase in resistance is due to specific acquired immunity has not yet been established Efforts have been made to study the relationship of allergy and immunity by desensitizing immune allergic guinea pigs during the course of experimental reinfection Local and general desensitization to tuberculin can be accomplished by the repeated injection of properly spaced increasing amounts of tuberculin into hypersensitive hosts (including man) Such experiments are difficult to perform Repeated injections of large amounts of tuberculin are toxic for normal animals and dramatic focal and general deleterious effects can be produced in tuberculous hosts particularly when too large amounts of tuberculin are injected into individuals who have not yet reached the proper degree of desensitization These investigations have been discussed at length by Rich (1951) The results of desensitization experiments strongly suggest that acquired resistance is not necessarily dependent on cutaneous or systemic hypersensitivity to tuberculin

On the other hand passive serum transfer studies as pointed out earlier have failed to demonstrate that the specificity of acquired resistance is mediated by a humoral mechanism

Lurie (1942) studied this problem in another way He studied the multiplication of tubercle bacilli in monocytes from normal and immune rabbits in the presence of normal and immune serum in the anterior chamber of the rabbit eye His results have been interpreted as evidence for a strictly cellular mechanism of acquired resistance independent of humoral antibodies This is probably unjustified in view of the observation that humoral antibody may appear locally at the site of transfer of washed mononuclear cells* from immunized donors to normal animals (Chase 1951) Suter (1953) Mackaness (1954) and Berthrong (1959) have studied the multiplication of tubercle bacilli in monocyte cultures in vitro suppressing extracellular multiplication of the parasites by streptomycin The results of their studies can be summarized as follows

1 When the number of virulent tubercle bacilli taken up by mononuclear phagocytes in vitro is small (no more than 5 to 10 cells per phagocyte) the phagocytes from immunized guinea pigs suppress intracellular multiplication while those of normal animals do not

2 This suppression of intracellular mul

* Mononuclear cells include all leukocyte types phagocytic and nonphagocytic except polymorphonuclear leukocytes

ulcerates and the ulcer usually does not heal The regional lymph nodes develop tubercles and caseate In contrast with this the already tuberculous guinea pig reacts differently to *superinfection* When after a period of a few weeks the infected animal is superinfected *in the same way* in the opposite thigh a dark colored induration develops within 2 days at the site of injection The skin over the indurated area undergoes necrosis and soon a superficial ulcer appears However this ulcer often heals quickly Infection of regional lymph nodes is retarded or fails to develop In other words the infection has brought about two changes the host has become hypersensitive to tubercle bacilli and it has acquired the capacity to localize the superinfection Acquired resistance to infection can best be shown by infecting guinea pigs first with a strain of low virulence (BCG for example) and later with a small number of virulent bacilli The progress of the virulent superinfection is much retarded but rarely entirely inhibited

Koch found that tuberculous guinea pigs react with inflammation not only to living but also to killed tubercle bacilli as well as to their protein fractions (Tuberculin) Later it was established that the Koch phenomenon can be demonstrated in guinea pigs sensitized by killed as well as by living tubercle bacilli Attempts to induce tuberculin sensitization of the delayed type with tuberculoprotein alone have been unsuccessful However it appears that the chloroform soluble wax fraction of tubercle bacilli which contains bound protein (or peptide) can confer the delayed type of tuberculin allergy on guinea pigs (Raffel 1948)

It would seem that the cells present in the tubercle play an essential role in the development of allergy to protein components of tubercle bacilli Certain aspects of this subject are dealt with in Chapter 11 particularly the difference between the tuberculin type and other types of hypersensitiveness

The Koch phenomenon has usually been considered a useful paradigm of immunity experiments because it takes place in the skin and is accessible to direct observation It has been assumed that the same events occur when tubercle bacilli are introduced into the lung by inhalation However it should be pointed out and emphasized that it is necessary to employ large numbers of living or killed tubercle bacilli to elicit the prompt inflammatory reaction and necrosis with ulcer formation which are seen in the classic *Koch phenomenon* Such reactions certainly do not take place under natural conditions of aerogenic infection with tubercle bacilli in experimental animals or in man Indeed there is no evidence of the killing effect of host resistance or hypersensitivity when the local infective (or reinfective) unit consists of one or a very few bacterial cells Under such conditions specific acquired resistance manifests itself only as a retardation partial or complete of the multiplication of the newly introduced tubercle bacilli

The tubercle its monocytes and epithelioid cells and the accelerated formation of tubercles have commonly been viewed as the means of defense of the host against the parasite but it would seem less prejudicial to look on them merely as anatomic evidence that changed reactivity of the host brings about conditions which limit local multiplication of the parasite

The significance of the role of allergy in immunity against tuberculosis has long been the subject of intense controversy At least 5 different viewpoints have been held on this complex problem

1 Specific acquired resistance to tuberculosis is a consequence of hypersensitivity to tuberculin and does not involve the participation of humoral antibodies against antigens of tubercle bacilli

2 Specific acquired resistance is due to humoral antibodies against one or more unidentified antigens of tubercle bacilli and is not in any way dependent on hypersensitivity to tuberculin

3 Specific acquired resistance is due to humoral antibodies and tuberculin hypersensitivity is an undesirable accompaniment of infection tending to interfere with the protective effect of humoral antibodies

4 Specific acquired resistance is due to humoral antibodies tuberculin hypersensitivity tends to have a favorable adjuvant effect on the operation of the specific humoral mechanism

5 Specific acquired resistance in tubercu

losis operates by an as yet undemonstrated cellular mechanism unlike any hitherto known to exist in other infectious diseases and dependent on neither humoral antibodies nor hypersensitivity to tuberculin

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tiplication by the immune phacogytes does not require the presence of serum from the immunized animal either before or after phagocytosis

Fong *et al* (1957) have observed that monocytes from immunized guinea pigs especially when cultivated in the presence of specific immune serum are protected against the primary cytotoxic effect of virulent tubercle bacilli which otherwise can be observed within 10 hours after intracellular infection However the degree of immunologic specificity of this protective function and its bearing on immunity as it is manifested in vivo are undefined (Elberg *et al*, 1957) Equally difficult to interpret are the observations of Sever (1960) demonstrating prolongation of the survival time of mice infected with large numbers of tubercle bacilli by the intravenous route after passive transfer of monocytes from immunized mice Indeed some experiments of Dubos and Schaedler (1956) indicate that some phenomena which have been interpreted as evidence of specific acquired immunity involve nonspecific tolerance mechanisms (as following injections of endotoxins)

It seems best to reserve further judgment on the subject of the specific and the nonspecific mechanism of acquired resistance to tuberculosis until further experimental data are available

In a highly infectious tuberculous environment the chance of contracting progressive clinical tuberculosis is higher in tuberculin-negative persons than in those who are already tuberculin positive (Ferguson 1946) This has been observed repeatedly among student nurses and in other groups Particularly convincing are the observations made in a hospital for the insane where the exposure was high and considerable numbers of inmates were tuberculin negative when admitted (Flahiff 1939) Such observations in man and immunization experiments in animals have encouraged the study of prophylactic active immunization of man On the other hand where the incidence of new infections is very low as in most parts of western Europe and America today the new cases of clinically active tuberculosis occur predominantly among those older individuals in the population who are already hypersensitive to tuberculin (Palmer and Shaw 1953)

IMMUNIZATION

In several species of experimental animals injection of killed tubercle bacilli moderately increases resistance to tuberculous disease This is clearly shown for example in rabbits (Opie and Freund 1937) With very small infective doses some of the immunized animals may escape the disease entirely the majority develop tuberculosis with a course slower than in the nonimmunized controls The observed degree of acquired resistance attainable with killed bacilli depends on many as yet ill-defined factors with regard to both the immunizing antigen and the mode of subsequent challenge There appears to be no strain specificity for the acquired resistance and bovine and human strains are able to cross immunize There is no evidence that the increase in resistance elicited by killed tubercle bacilli is qualitatively different from that which can be achieved by living attenuated organisms Indeed under some experimental conditions killed bacilli have been observed to be as effective as living attenuated organisms (Dubos *et al* 1953) Nevertheless living attenuated bacilli are more regularly effective than are killed bacterial cells The question of the relative duration of acquired resistance induced by killed bacilli compared with living bacilli as well as chemical fractions thereof has not been adequately investigated

The immunizing effect of heat killed tubercle bacilli in man was studied in a hospital for the insane by inoculating alternate tuberculin negative patients within 2 weeks after admission (Opie *et al* 1939) * The morbidity and mortality rates during the subsequent 18 months were significantly lower in the vaccinated than in the unvaccinated group

There is convincing evidence that vaccination with living strains of attenuated tubercle bacilli derived from the original attenuated strain of Calmette and Guerin (BCG) can confer some protection against

* Only tuberculin negative individuals are vaccinated with living or dead tubercle bacilli because of the Koch phenomenon which occurs in individuals who are already tuberculin positive

naturally acquired tuberculous disease in man (Aronson 1948 Hyge 1956) The degree of protection afforded by BCG vaccination can be estimated from the report of Dahlstrom and Difs (1951) on Swedish soldiers with a moderate exposure to infection and from a recent report of the British Medical Research Council The evidence derived from the latter study indicates that BCG vaccination can confer significant protection not only against primary tuberculosis but also against the postprimary or reinfection type of disease (Med Res Council 1963)

Striking differences in invasiveness and immunizing capacity exist among the various BCG strains derived from the original culture (Jacox and Meade 1949 Suter and Dubos 1951 Dubos *et al* 1956) Several groups of investigators claimed in the early years of its use that BCG could dissociate back to a virulent variant but there is no authenticated case of progressive and fatal tuberculosis in human beings attributable to such a spontaneous mutation of the vaccine On the other hand at least 4 well authenticated cases of progressive tuberculosis in man attributable to BCG have been reported in individuals with peculiarly inadequate ability to develop or maintain resistance to tuberculous infection (Ustvedt 1956) That BCG strains are usually less attenuated than the completely non cord forming variants of tubercle bacilli (e g H37Ra) is evident from the fact that at least 2 BCG strains have been found to cause progressive and fatal tuberculosis in silicotic guinea pigs (Vorwald *et al* 1950) whereas certain still more attenuated strains are unable to cause disease under these conditions

No basically novel technic has been introduced in practice to improve on Calmette s original method of preparing the vaccine in spite of the fact that large variations in activity occur from one preparation to another (Fenner 1951 Centre International de l Enfance 1955) However a technic of aerogenic infection has been employed to bring out striking differences in the infectivity of various daughter strains of BCG (Middlebrook 1961) Furthermore these studies have shown that infection with as few as 10 viable units of one BCG strain by inhalation sufficed to provide as much immunity as 1 000 000 bacterial cells of the same strain introduced by the cutaneous route in guinea pigs

It is possible that not only the living but also the dead bacterial cells in a BCG vaccine contribute to the development of tuberculin hypersensitivity and perhaps also to the resistance conferred by BCG vaccination

Active immunization with BCG or with other attenuated strains (for example the vole bacillus) or killed bacilli has some usefulness in protecting tuberculin negative individuals However it should be recalled that persons with healed tuberculous lesions can acquire new exogenous reinfection and progressive disease Artificial immunization with the vaccines thus far offered is not likely to be more effective than previous infection with virulent tubercle bacilli Nonetheless it is desirable to have available a prophylactic vaccine such as BCG where its use is indicated (Rosenthal 1957) More than 200 000 000 persons have been tuberculin tested and 75 000 000 vaccinated with BCG under the auspices of the World Health Organization

The vole bacillus is capable of inducing in guinea pigs a degree of active immunity comparable with that produced by BCG Experimental immunization of human beings with vole bacilli has been undertaken by the British Medical Research Council in the belief that a spontaneous change in pathogenicity of this type of tubercle bacillus for man is less likely than a spontaneous increase in virulence of the attenuated BCG strains (Wells 1946) A reasonable degree of acquired resistance to clinical tuberculosis has been observed comparable with that provided by BCG

Chemotherapy

The antimicrobial drug treatment of ulcerated tuberculosis has provided an unparalleled opportunity to study a host drug parasite relationship in clinical medicine It presents most of the problems encountered in general in chemotherapy of infectious processes and yet the response to treatment is in slow motion as it were in contrast with the situation in the acute infectious processes caused by more rapidly multiplying parasites

Three chemotherapeutic agents are in common clinical use at the present time for the treatment of tuberculosis streptomycin para aminosalicylic acid (PAS) and isoniazid (INH) Streptomycin exerts its therapeutic effect by bacteriostatic activity at low concentrations and bactericidal activity at higher concentrations Therapeutic benefit can be achieved with this drug alone in pulmonary tuberculosis genitourinary tuberculosis, miliary tuberculosis tuberculous meningo encephalitis and in the preparation of patients for thoracic surgery The degree of benefit is dependent on many factors too complex to recount here Suffice it to state that the results attainable are conditioned by the extent of ulceration of the tuberculous lesions (e g number and size of pulmonary cavities) and the speed with which necrosis is occurring at the time of initiation of chemotherapy Thus the factors which determine the effectiveness of chemotherapy with streptomycin in tuberculosis are similar to those involved in chemotherapy of other infections in which tissue necrosis is a significant part of the disease process (McDermott, 1949) The acute exudative lesion often responds dramatically while the large necrotic or partially necrotic lesions are controlled less readily and may persist to form the basis for further extensions of the disease after the cessation of chemotherapy

Tubercle bacilli like other susceptible bacteria tend to mutate to individuals resistant to streptomycin both in artificial cultures and in the tuberculous host Since in tuberculosis, one is often coping with very large populations of parasites streptomycin fastness is a conspicuous problem Furthermore it has been observed that streptomycin resistant tubercle bacilli can infect other persons This factor, as well as the toxicity of streptomycin for the vestibular and the auditory functions has placed marked limitations on the useful ness of this drug alone in the chemotherapy of tuberculosis

PAS exerts a bacteriostatic but little or no bactericidal effect on tubercle bacilli in vitro and has limited but recognizable chemotherapeutic activity in human disease (Therapeutic Trials Committee 1950) It is much more active against the mammalian types of tubercle bacilli than against any other microbes thus far tested Its antibacterial activity is competitively antagonized by low concentrations of para aminobenzoic acid

In 1952 the third drug of the triumvirate isonicotinic acid hydrazide (isoniazid), was discovered to have potent antimicrobial activity against tubercle bacilli in vitro in experimental animals and in man Subsequently this compound has been found to have properties which clearly make it the most important antimicrobial chemotherapeutic agent for tuberculosis Its activity is strictly limited to tubercle bacilli it is much less active against other mycobacteria—probably useless in leprosy for example Isoniazid is active in vitro against over 99 per cent of strains of typical tubercle bacilli at levels of 0 02 to 0 2 mcg /ml and against one of the commonest types of atypical tubercle bacilli the yellow bacilli at 0 2 to 1 0 mcg /ml Claims have been made that some wild strains of typical tubercle bacilli are more resistant than this but such strains must be exceedingly rare

Isoniazid unlike streptomycin and PAS is active against intracellular tubercle bacilli Whereas streptomycin has some sterilizing activity for tubercle bacilli provided with a carbon source but no nitrogen source or no oxygen isoniazid in contrast is sterilizing for these organisms only when they are actually multiplying

In addition to the first line drugs streptomycin PAS and isoniazid certain other antimicrobial agents also are used in treatment particularly of patients whose bacterial populations are already resistant to the first line drugs Among these second line drugs are pyrazinamide cycloserine ethionamide kanamycin viomycin and capreomycin Pyrazinamide exerts its sterilizing antibacterial effects only in acidic environments in artificial media (McDermott and Tompsett 1954) and in monocyte cultures (Mackaness 1956) The combination pyrazinamide and isoniazid exerts the greatest chemotherapeutic activity of any of the drug combinations thus far studied in experimental infections of mice (McCune *et al* 1956)

As with streptomycin the mutation rate of tubercle bacilli to isoniazid resistance is high enough so that in nearly 50 per cent of isoniazid treated tuberculous patients with

ulcerative tuberculosis of the lungs isoniazid resistant mutants make their appearance in large numbers in the sputum if this drug is used alone In fact over 90 per cent of patients who remain bacteriologically positive after 4 months of treatment with high dosage of isoniazid (8 to 16 mg /Kg /day) excrete bacilli of which a great proportion are highly resistant to the drug (to 10 or more mcg isoniazid/ml)

Two distinct *types of isoniazid resistant mutants* have been recognized catalase positive and catalase negative All isoniazid-susceptible human and bovine strains of tubercle bacilli have catalase activity varying quantitatively over a fairly narrow range Catalase positive isoniazid resistant mutants can be isolated from most isoniazid susceptible populations when these are exposed to concentrations of isoniazid just above the minimal antibacterial concentration of this drug One step mutation to resistance to higher concentrations of isoniazid (1 or more mcg / ml) without complete loss of catalase is a very rare event On the other hand one step mutation to high resistance to isoniazid (10 or more mcg /ml) with complete loss of catalase is common The prevalence of such mutants is 1 in 10^4 to 1 in 10^6 in most populations of tubercle bacilli (Middlebrook 1956)

Isoniazid is metabolically altered in the human body to chemical derivatives which have little or no antimicrobial activity (Hughes 1953) and this biochemical alteration the first step of which is probably acetylation occurs at widely different rates in different individuals though this rate remains relatively constant in any one person over long periods of time A correlation has been established between the rate of metabolic inactivation of isoniazid as determined by microbiologic assay of serum levels of the antimicrobially active drug and the types of resistant mutants—catalase positive or catalase negative—which emerge in patients with pulmonary tuberculosis under treatment with isoniazid (Mandel *et al* 1957) Thus serum assay for isoniazid may be used to estimate the concentrations of drug delivered to the multiplying tubercle bacilli in open tuberculous cavities and there is some evidence to suggest that success of chemotherapy with this drug depends in part on the serum level of active drug and hence on the dose administered to the individual patient

The biochemical implications of loss of catalase activity for the mechanism of loss of isoniazid susceptibility are not clear However it has been established that catalase deficiency almost always results in decreased pathogenicity of tubercle bacilli While very large doses of catalase negative isoniazid resistant mutants can multiply sufficiently during the first few weeks of primary infection to cause death small inocula are unable to cause the inexorably progressive disease characteristic of virulent isoniazid susceptible strains Thus catalase negative strain can be highly *infective* in spite of their diminished pathogenicity

Catalase-deficiency is also accompanied by increased susceptibility of the bacilli to the toxic effects of exogenous H_2O_2 although one anomalous catalase negative strain has been found to be quite resistant to H_2O_2 and interestingly enough fully pathogenic Thus it has been observed that the diminished pathogenicity of isoniazid resistant strains is intimately related to their H_2O_2 hypersusceptibility Therefore it has been suggested that the concentration of hydroperoxides in phagocytic cells increases during the development of host responsiveness in tuberculous infection (Middlebrook 1956)

Combined Drug Therapy It was early observed that the simultaneous presence of streptomycin and another unrelated antibacterial agent in culture media prevented the appearance of streptomycin resistant mutants of tubercle bacilli in vitro (Middlebrook and Yegian 1946) It has also been established that clinical use of streptomycin or PAS along with isoniazid usually prevents the appearance of drug resistant tubercle bacilli in treated patients (Tuberculosis Chemotherapy Trials Committee 1955 U S Veterans Administration—Armed Forces 1957) Thus it has become common practice to use simultaneously *at least 2 drugs on initiation of treatment of clinically active tuberculosis*

For the best chemotherapeutic result it is necessary that the population of tubercle bacilli be characterized as to its susceptibility to the antibacterial drugs which may be used

Three chemotherapeutic agents are in common clinical use at the present time for the treatment of tuberculosis streptomycin para aminosalicylic acid (PAS) and isoniazid (INH) Streptomycin exerts its therapeutic effect by bacteriostatic activity at low concentrations and bactericidal activity at higher concentrations Therapeutic benefit can be achieved with this drug alone in pulmonary tuberculosis genitourinary tuberculosis miliary tuberculosis tuberculous meningoencephalitis and in the preparation of patients for thoracic surgery The degree of benefit is dependent on many factors too complex to recount here Suffice it to state that the results attainable are conditioned by the extent of ulceration of the tuberculous lesions (e g number and size of pulmonary cavities) and the speed with which necrosis is occurring at the time of initiation of chemotherapy Thus the factors which determine the effectiveness of chemotherapy with streptomycin in tuberculosis are similar to those involved in chemotherapy of other infections in which tissue necrosis is a significant part of the disease process (McDermott, 1949) The acute exudative lesion often responds dramatically while the large necrotic or partially necrotic lesions are controlled less readily and may persist to form the basis for further extensions of the disease after the cessation of chemotherapy

Tubercle bacilli like other susceptible bacteria, tend to mutate to individuals resistant to streptomycin both in artificial cultures and in the tuberculous host Since in tuberculosis one is often coping with very large populations of parasites streptomycin fastness is a conspicuous problem Furthermore it has been observed that streptomycin resistant tubercle bacilli can infect other persons This factor as well as the toxicity of streptomycin for the vestibular and the auditory functions has placed marked limitations on the useful ness of this drug alone in the chemotherapy of tuberculosis

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In 1952 the third drug of the triumvirate isonicotinic acid hydrazide (isoniazid), was discovered to have potent antimicrobial activity against tubercle bacilli in vitro in experimental animals and in man Subsequently this compound has been found to have properties which clearly make it the most important antimicrobial chemotherapeutic agent for tuberculosis Its activity is strictly limited to tubercle bacilli it is much less active against other mycobacteria—probably useless in leprosy for example Isoniazid is active in vitro against over 99 per cent of strains of typical tubercle bacilli at levels of 0 02 to 0 2 mcg /ml and against one of the commonest types of atypical tubercle bacilli the yellow bacilli at 0 2 to 1 0 mcg /ml Claims have been made that some wild strains of typical tubercle bacilli are more resistant than this but such strains must be exceedingly rare

Isoniazid unlike streptomycin and PAS is active against intracellular tubercle bacilli Whereas streptomycin has some sterilizing activity for tubercle bacilli provided with a carbon source but no nitrogen source or no oxygen isoniazid in contrast is sterilizing for these organisms only when they are actually multiplying

In addition to the first line drugs streptomycin PAS and isoniazid certain other antimicrobial agents also are used in treatment particularly of patients whose bacterial populations are already resistant to the first line drugs Among these second line drugs are pyrazinamide cycloserine ethionamide kanamycin viomycin and capreomycin Pyrazinamide exerts its sterilizing antibacterial effects only in acidic environments in artificial media (McDermott and Tompsett 1954) and in monocyte cultures (Mackaness 1956) The combination pyrazinamide and isoniazid exerts the greatest chemotherapeutic activity of any of the drug combinations thus far studied in experimental infections of mice (McCune *et al* 1956)

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Curiously enough there is no striking evi dence in experimental animals that age has an influence on the progress of the infection but in man it is undoubtedly true that pri mary tuberculosis in infants has a worse prognosis than in children or adults The death rate varies with sex and age Tuber culosis in all race sex groups in the United States is becoming a disease of the older people Nevertheless for the United States as a whole half of the newly reported active cases in 1956 were under 43 years of age The differences between men and women can be due both to basic physiologic differ ences associated with sex and to different degrees of exposure and living conditions Physiologic factors undoubtedly play a major role sex differences in susceptibility have also been observed in mice especially after BCG immunization (Hoyt *et al* 1957)

It is generally believed that malnutrition increases susceptibility to tuberculosis The increase in morbidity and mortality rates dur ing wars in some countries has been attrib uted to concurrent malnutrition Overcrowd ing and other factors were also present and their relative significance is difficult to eval uate (Dubos and Dubos 1952) It has not yet been convincingly shown that known vitamins or caloric intake have an influence on tuberculous infection in man or experi mental animals However some nutritional and metabolic factors have recently been found to affect the course of tuberculous in fection in mice (Dubos 1955) and in mice and hamsters at least protein intake is of great importance (Schaedler and Dubos 1956 Ratcliffe and Merrick 1957)

The course of tuberculous infection is in fluenced by occupation Exposure to tubercle bacilli is frequent in the nursing and the med ical professions Inhalation of dust contain ing silica (silicon dioxide) in certain trades such as granite-cutting and certain types of

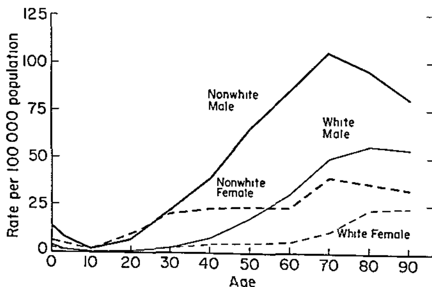


FIG 6 Age specific tuberculosis death rates by race and sex for the United States in 1956 (From 1957 Tub rculosis Chart Series U S Dept of Health Education and Welfare Division of Special Health Services)

This is particularly important if there is any possibility that the patient has been treated previously with one or more of these drugs or has been exposed to individuals excreting drug resistant mutants

In practice if more than 1 per cent of the population of tubercle bacilli available for drug susceptibility testing are resistant to 2 to 5 mcg of streptomycin per ml of culture medium this drug is not likely to be fully effective The analogous concentration for isoniazid is 0 2 to 1 0 mcg and for para aminosalicylic acid 2 to 5 mcg per ml On the other hand if the population of tubercle bacilli harbored by a patient is susceptible to both streptomycin and isoniazid and these two drugs are given simultaneously and uninterruptedly in adequate dosage for a sufficient period of time there is every reason to anticipate a successful chemotherapeutic response in over 98 per cent of cases (Russell and Middlebrook 1961) A final evaluation of intensive combined drug approach to chemotherapy of tuberculosis cannot be anticipated in view of the possible obstacle posed by the resting tubercle bacilli—so called persistors —which escape sterilization because of their phenotypic insusceptibility to drug action Thus there is no evidence that any chemotherapeutic regimen thus far devised for tuberculosis is truly eradicative except under uncommon circumstances

In recent years there have been many reports of a rising incidence of primary infection with drug resistant strains Information available at present especially with regard to isoniazid resistance does not allow any conclusions about the magnitude of this problem or its possible significance for the epidemiology and the therapy of tuberculosis in the future (Mitchison 1962)

Prophylactic use of chemotherapy has been suggested for (1) preventing primary infection and (2) preventing latent infection from evolving into manifest disease The administration of antimicrobial drugs for either of these separate purposes is beset by many practical problems not the least of which is motivating a healthy human being to take medications for many weeks or months Nevertheless there is evidence that the administration of isoniazid can prevent a primary infection from taking under certain experimental conditions (Cohn *et al* 1962), and there is clinical evidence that antimicrobial treatment of primary infection in the highly susceptible infant is effective in preventing the early complications of the primary complex (Robinson and Meyer 1956 Ferebee and Mount 1962) However the fact that isoniazid the agent which might practically be applied in prophylactic chemotherapy is unable to sterilize resting tubercle bacilli presents an obstacle the significance of which can be defined only by experience (Mitchison 1957) Cooperative investigations of this problem are already under way in France and in the United States (Debre 1956 Ferebee 1956) In any case programs of control of tuberculosis by chemotherapy cannot be considered to exclude immunization because the wide variations in the exposure rate in the world today provide room for either approach depending on many factors including the epidemiologic and the ecologic situation of the individual or group

EPIDEMIOLOGY

Within several species of animals certain experimentally inbred families are consistently more susceptible than others (Lewis and Loomis 1928 Lurie 1941) The physiologic factors manifesting these genetic differences have been studied but not yet clarified

In man the role of genetic factors in susceptibility is illustrated best in the study of tuberculosis in homozygotic and heterozygotic twins (Kallmann and Reisner 1943) If one homozygotic twin has clinical tuberculosis the other twin has 3 chances in 4 of being affected also whereas if one heterozygotic twin has the disease the other has only 1 chance in 3 of developing clinical tuberculosis Studies aimed at the demonstration of familial differences in susceptibility (Puffer 1944) are difficult to interpret because of the complicating variable of amount of exposure

The incidence and the type of disease are different in the American Indian the Negro and the white races the former two appearing to be more susceptible (Opie *et al* 1936) (Fig 6) These differences are diffi

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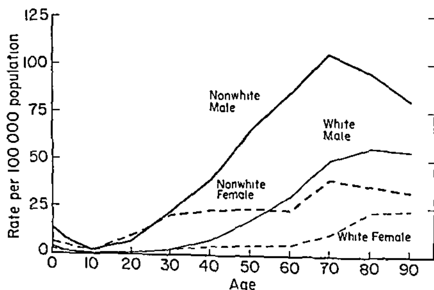


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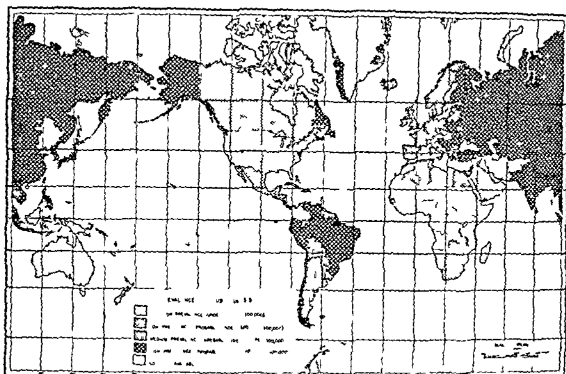


Fig 7 Prevalence of tuberculosis as estimated from the probable death rates from all forms of tuberculosis in the various countries of the world (Yelton S F 1946 Tuberculosis throughout the world Pub Health Rep *61* 1144 1160)

mining increases the susceptibility to progressive pulmonary disease *This is an important industrial health problem* In experimental animals the pathologic changes induced by silica accelerate the disease in *pulmonary and extrapulmonary* sites wherever silica and tubercle bacilli are associated Silica has no direct effect on the parasite its local toxic action on the tissue of the host in some way promotes the disease

The distinction between infection and disease is very significant in the epidemiology of tuberculosis The infection is more widespread in urban than in rural populations The risk of infection has been declining in all parts of the United States and is now estimated to be less than 5 new infections per 1 000 young adults per year at the present time in some areas it is higher and in others it is less than 1 new infection per 1 000 young adults per annum Still in 1962 there were estimated to be at least 35 million living Americans who had been infected with tubercle bacilli At the end of the calendar year 1960 there were 58 000 hospitalized cases of tuberculosis and 62 000 unhospitalized *cases in the United States* with an additional 50 000 other persons under drug therapy (U S P H S 1963) Thus disease at any given time in the clinically progressive *form is present* only in a small percentage of persons infected with tubercle bacilli Chronic tuberculosis in individuals over the age of 50 is often not recognized before necropsy

Tubercle bacilli of human type are transmitted from person to person almost exclusively by airborne droplet nuclei of sputum Bacilli of the bovine type reach man usually by way of unpasteurized milk from the tuberculous cow they also can be transmitted by the same route as the human type airborne Important aspects of the infection essential for the understanding of the epidemiology of the disease are

1 Infectious droplet nuclei containing tubercle bacilli originate from persons with ulcerated tuberculous lesions of the airways however small such ulcers or cavities may be

2 Not all infections result in disease

recognizable by roentgenograms or other clinical means Household contact is an important factor in infection

3 The disease may have an acute flulike onset followed many months or years later by excavation of necrotic pulmonary tissue cavitation and chronic phthisis

4 In some individuals it may be fairly advanced with cavity formation and large numbers of tubercle bacilli in the sputum and produce scant if any specific clinical symptoms As carriers these individuals spread the infection

5 The progress and the type of infection are influenced by many factors such as age and previous exposure Recovery from one attack of clinical disease does not result in solid immunity

6 The reinfection type of tuberculosis commonly seen in adults is sometimes exogenous (see Pathology)

7 Microepidemics in schoolrooms and school buses are becoming increasingly recognized as important epidemiologic patterns of tuberculous infection (Mahady 1961)

Tuberculosis has been known in urban civilization since the beginning of recorded history In western civilization the death rate has been declining since 1870 *i e* before the discovery of the parasite While no longer among the leading causes of death in the United States tuberculosis is still the most frequent cause of death between the ages of 15 and 45 years and probably the principal cause of death in the world The fact that many individuals young and old are incapacitated for long periods of time by tuberculosis although they may not die of it gives it great social and economic significance The ratio of newly discovered active cases to deaths per year in the United States is about 6 to 1 The decrease in mortality rate from tuberculosis has not been paralleled by an equal decrease in morbidity and prevalence of infection It appears likely that the present rate of decline in mortality will continue as a result of chemotherapy but morbidity will surely decline more slowly In the Orient in Central and South America in Africa and in Asia both morbidity and mortality rates are still very high (Fig 7)

Control Measures

McPhedran and Opie (1935) wrote The spread of tuberculosis occurs in large part by long drawn-out family or household epidemics in which the disease is slowly transmitted from one generation to the next For many decades this observation served as a basis for most of the measures used in the control of tuberculosis However during the past decade it has become increasingly recognized that the ecology of tuberculosis has changed It has changed in three ways The epidemics are no longer long and drawn-out Family and household contacts still account for the major proportion of new infections in children However in adults new infections are not so commonly familial as they were some years ago And finally the expression the disease is slowly transmitted is inconsistent with contemporary knowledge of the epidemiology of tuberculous infection and disease

Perhaps the most important single aspect of the infection (which must always precede the disease) is the fact that it is initiated today by a single droplet nucleus directly from the nebulizing respiratory tract of an individual who has large numbers of tubercle bacilli in the sputum If nebulization is ineffective—lack of sufficiently forceful cough or sneeze—or if there are few or no tubercle bacilli in the sputum a new infection will not be established in another individual Other variables are involved too such as the nature and the quantity of ventilation of the environment which influence the behavior of the potentially infectious droplet nucleus

Although the reasons for carrying out certain measures in the control of tuberculosis have changed nevertheless the measures themselves have changed only quantitatively This applies to the control of tuberculous infection and disease in populations in general and especially to so-called captive populations such as schools hospitals military personnel industrial personnel and government employees The following are the important steps in control of tuberculosis

1 Prevent infection.

2 If infection does occur detect it and its source as certainly and as early as possible

3 Treat the young infection with one or more antimicrobial drugs

4 If infection has been initiated at some unknown time more than one year prior to its first being detected look for and continue to monitor for active disease

5 Treat active disease effectively with the aim of complete rehabilitation

These theoretic ideal goals are not and perhaps, cannot be perfectly achieved in practice However it will be useful for the continuation and the development of operational procedures to analyze practical approaches with these goals in mind

The first goal—prevention of infection—is approached practically from two standpoints control of potential sources of infection on the one hand and on the other protection of the exposed individual The isolation of the actual or the potential spreader was the first widely applied public health measure after the discovery of tubercle bacilli by Robert Koch In practice however even today isolation means different things to different people Recent studies of Riley *et al* (O Grady and Riley 1963) at Johns Hopkins re-emphasize the importance of air borne droplet nucleus directly from the individual with large numbers of tubercle bacilli in the sputum as the sole source of the infectious agents of tuberculosis in modern civilization And they re-establish the improbability of contact infection clearly distinguishing the mode of transmission of tuberculous infection from the commonly accepted mode of transmission of enteric infections

Preoccupation with the careful disposal of physical articles in the environment of the patient sterilization of eating utensils and disinfection of articles which may be contaminated by fomites containing tubercle bacilli seem to be unjustified in the context of what is presently known concerning the mode of transmission and introduction of tubercle bacilli to the susceptible locus in the human subject The susceptible locus seems to be the pulmonary spaces proximal to the ciliary mucous escalator Unlike potentially infectious droplet nuclei with aerodynamic dimension less than 5 microns in diameter dust particles from fomites appear to be non infectious simply by virtue of the fact that their size is very rarely as small

With regard to the protection of exposed individuals, the advisability of the use of vaccination or other immunization procedures deserves to be seriously considered There seems to be no doubt that vaccination with BCG, under the best conditions provides up to 80 per cent protection against tuberculous disease Data from experimental animal studies as well as from clinical observations on human beings would appear to indicate that vaccination with BCG does not protect against *infection* under natural conditions with virulent tubercle bacilli Many authorities have expressed the opinion that in the United States the advantages of the vaccination are outweighed by the disadvantages of obscuring the usefulness of th tuberculin test for continued epidemiologic monitoring especially in the face of the relative effectiveness of chemoprophylaxis of young infections and multiple drug treatment of established active disease

With regard to the *detection* of infection the tuberculin skin test still has great merit in spite of the interfering effect of the consequences of infection with mycobacteria other than *M tuberculosis* Detection plays the major role in present day programs of control of tuberculosis where the incidence of infection is low as is the case now in the general population of the United States

It now seems feasible and economical to test with tuberculin school children and as many individuals in other population groups as possible and to repeat skin testing in connection with the normal cycling of other general medical and immunologic procedures permitting continued monitoring of the epidemiologic situation

In *microepidemics* the source should be vigorously sought and it is usually not difficult to find On the other hand sources of new infections contracted in high incidence areas of the United States or of the world are usually very difficult to identify

Finally it seems important to stress treatment or prophylaxis of infection aimed at preventing infection from becoming overt disease The United States Public Health Service studies have clearly shown that this is possible Furthermore the use of one drug normally isoniazid seems sufficient especially in consideration of the disadvantages and the expense of using more than this

drug alone for such purpose The customary period of recommended administration of isoniazid for chemoprophylaxis is one year in a single dose per day Some authorities do not believe that it need be so long but information is insufficient to define the mini mum effective period The principal difficulty encountered in this area is in deciding whether or not to treat an individual with clearly positive tuberculin hypersensitivity when the duration of time between the con traction of infection and the observation of tuberculin hypersensitivity is unknown but is more than a year or so This matter is controversial and is likely to remain so

Some authorities administer isoniazid to individuals who are predisposed to the de velopment of tuberculous disease such as diabetics individuals receiving steroids genetically predisposed individuals etc reasoning that possible benefits of isoniazid outweigh the risk of toxicity

The ideal program of tuberculosis control must always emphasize continued training of physicians nurses bacteriologists pathol ogists radiologists and technicians The extrapolation of curves now available in regard to the changes in the incidence of new infections and of active disease suggests a much slower disappearance of tuberculosis than was originally foreseen following intro duction of effective therapy

It is difficult to evaluate the comparative effectiveness of the conscious public health measures of the last 60 years and the rise in the standard of living in combating tuber culosis in the United States and Europe It seems probable that improved economic status with less crowding in households better ventilation better nutrition and better education as to personal hygiene during this century has had a very large share in di minishing the prevalence of tuberculous in fection and disease

TUBERCULIN

Old Tuberculin (O T) is prepared in the following manner Tubercle bacilli are grown on a glycerine broth or a completely synthetic medium for 4 to 6 weeks the cul tures are steamed at 100 C for a few hours and then evaporated to one tenth of the original volume and passed through a filter to remove the bacteria The order of evap orating and filtering may be reversed It is possible to obtain fractions of the culture filtrates of tubercle bacilli possessing higher tuberculin activity per unit of dry weight than such crude preparations Tuberculin activity is associated with the protein fractions of tubercle bacilli (see Chemical Constituents of Mycobacteria) Now widely used are fractions of culture filtrates of tubercle bacilli called PPD (purified protein derivative) obtained either by trichloroacetic acid or half saturated ammonia sulfate precipitation No preparations of tuberculoproteins have been obtained free of polysaccharide and nucleic acid Both the old tuberculin and PPD preparations have to be standardized in guinea pigs or man since biologic activity varies with different lots *

Tuberculins made with human or bovine strains and from *M kansasii* and *M balnei* cannot be distinguished from each other but are quite different from avian tuberculin PPD preparations from Battey strains as well as from pathogenic scotochromogenic strains are sufficiently different from similar preparations from tubercle bacilli to permit their use in clinical differentiation of these infections (Edwards and Edwards 1960)

For the Mantoux or intradermal skin test one tenth of a milliliter of a high dilution of tuberculin is injected into the skin over the forearm When active tuberculous disease is definitely suspected it is customary to use first a small dose 0 01 mg (0 1 ml of 1 10 000 dilution) and then if there is no reaction a larger dose namely 1 mg (0 1 ml of 1 100 dilution) The PPD prepara tion is used in amounts which produce re actions corresponding in intensity to 0 01 mg (first strength) 0 05 or 0 1 mg (in termediate strength) and 1 mg O T (second strength) The intermediate strength is commonly recommended for mass surveys today It is the equivalent of 5 or 10 international tuberculin units (T U) The tuberculin reaction is characterized by delayed appearance and relatively long du ration It usually appears several hours after injection and the maximum response may be seen in 1 or 2 days The reaction is con sidered as definitely positive if there is in duration more than 10 mm in diameter

* One standardized lot of PPD is commercially available for clinical use at the present time

erythema is usually present but is not considered in judging the size of the reaction in white persons it is commonly graded according to the longest diameter of the area indurated A papule or vesicle may develop in the central part of the indurated area and necrosis may occur here Weak reactions may appear and disappear faster than the stronger reactions they may be missed if the skin test is not read on the day following injection No control is necessary for the intracutaneous tuberculin test

Other forms of skin testing have been suggested to avoid the use of injections The oldest is the von Pirquet scratch test which consists of rubbing tuberculin into a scratched area tuberculin may be incorporated into an ointment or used as a patch test (Vollmer patch) More recently the Heaf gun (Andersen and Smith 1960) and the tine technic (Rosenthal 1961 Badger *et al* 1963) have been introduced particularly for convenience and economy in mass tuberculin testing surveys These methods of skin testing are less quantitative than the intracutaneous test

A positive tuberculin test indicates that the reactor has been infected but it does not necessarily indicate disease it is often positive in the absence of lesions recognizable by roentgenograms Lack of reaction indicates absence of infection with the following qualifications the person may be in the preallergic state during the early stage of first infection (a period not exceeding a month) or he may have lost allergy due to overwhelming tuberculous infection or to unrelated infections especially measles The latter is uncommon In general persons with active lesions and those exposed recently and often (household contacts) react to smaller doses of tuberculin than others (Furcolow *et al* 1941) Furthermore the intensity of a positive reaction in an individual who presents no additional evidence of active disease has some prognostic significance since it has been oberved that those who react more strongly are more likely to develop clinically demonstrable disease in the future The tuberculin test is of great value in excluding tuberculosis and also in epidemiologic studies to indicate the prevalence of infection Loss of tuberculin sensitivity in persons with healed tuberculosis of first infection has been observed repeatedly in recent years, and it can be induced in experimental animals by chemotherapy Casual contacts with tubercle bacilli are becoming less frequent Thus one factor in conditioning the maintenance of tuberculin allergy is becoming less prevalent Tuberculin skin sensitivity is often lost in persons who develop Boeck s sarcoid (Reisner 1944)

False positive reactions to the larger doses of tuberculin (50 to 250 T U) occur in certain geographic areas notably across the southern part of the United States in Egypt and in certain parts of India (Palmer 1953) It seems probable that the great majority of weak positive reactions given by persons residing in the southeastern part of the United States are attributable to infection with Battey strains (Edwards *et al* 1959) The existence of such false positive reactions to tuberculin must be recognized if a true picture of the epidemiology of tuberculosis is to be obtained

When a large amount of tuberculin 0 5 ml of undiluted O T for instance is injected into a tuberculous guinea pig a systemic reaction ensues quite gradually the tubercles and the surrounding tissues become congested and often hemorrhagic exudate appears in the peritoneal and the pleural cavities and the animal may die in 24 to 72 hours What relationship this reaction bears to tuberculin allergy of the skin is not clear Anaphylactic symptoms usually fail to appear Systemic tuberculin reactions may also be produced in hypersensitive human beings and untoward effects may be produced with excessive doses in ocular tuberculosis and tuberculous cervical adenitis but the previously disastrous consequence of such reactions can be controlled by cortisone and antimicrobial chemotherapy

As already discussed hypersensitivity of the skin to tuberculin indicates infection but not necessarily disease A negative test usually rules out infection A positive tuberculin reaction in an infant indicates progressive disease requiring antimicrobial chemotherapy because of their high susceptibility Conversion from negative to positive signifies recent exposure and infection frequent examinations of such cases are indicated especially

because chemotherapy with isoniazid may prevent the subsequent development of clinical disease (see Chemotherapy)

Laboratory Procedures

Bacteriologic Methods

The bacteriologic diagnosis of tuberculosis rests on the demonstration of tubercle bacilli in the sputum or other pathologic material from a patient A few years ago the laboratory diagnosis of tuberculosis could be made on the basis of the finding of acid fast bacilli in a suitably stained smear of the material submitted to the laboratory Today it is desirable if not essential to culture routinely all specimens submitted for examination for acid fast bacilli With adequate culture methods the more expensive procedure of animal inoculation is rarely needed for diagnostic purposes In addition to being a much more sensitive method for detecting acid fast organisms than either direct or concentrated smear the culture technic provides essential information on the properties of the infecting organism which are relevant to therapy

At this time approximately 98 per cent of human pulmonary mycobacterioses in the United States are caused by the classic human type tubercle bacillus The remaining 2 per cent of human infections have been shown recently to be caused by other species of mycobacteria In some areas of this country the incidence of infections caused by these unclassified acid fast organisms may be as high as 10 per cent

It is necessary for these infectious agents to be identified precisely in the laboratory because the prognosis and the response to drug therapy differ greatly for infections caused by each individual type of mycobacterium All mycobacteria are acid fast and it is usually not possible to differentiate typical tubercle bacilli from the other unclassified mycobacteria on the basis of a Ziehl Neelsen stain alone In Table 1 are shown some of the differential characteristics of the more frequently encountered mycobacterial species associated with pulmonary disease With use of these cultural differences and the appropriate laboratory procedures most mycobacteria can be identified precisely within 3 to 4 weeks after submission of the specimen to the laboratory (Middlebrook and Cohn 1958 Veterans Administration 1962)

In addition to routine cultures the clinician treating tuberculosis today should have drug susceptibility studies made on the acid fast organisms isolated from the patient A few years ago it was customary to obtain this information by first isolating the mycobacteria on egg medium then emulsifying or subculturing the growth and finally inoculating a series of tubes of suitable culture medium containing graded concentrations of selected antituberculosis drugs This so-called indirect drug susceptibility test required 6 to 10 weeks from the time the original sputum specimen was submitted to the laboratory It is practical to inoculate decontaminated clinical material submitted for culture directly on medium containing clinically useful concentrations of primary drugs (isoniazid streptomycin and para amino salicylic acid) This is done conveniently by the use of Felsen quadrant petri dishes and 7H 10 medium (Russell and Middlebrook 1961) This direct method routinely provides culture reports and primary drug susceptibility results in 3 weeks

The widespread use of the primary antituberculosis drugs during the past 15 years has caused the selection of isoniazid and/or streptomycin resistant strains of tubercle bacilli in many patients who were improperly treated with these drugs Obviously primary chemotherapy regimens no longer will help these patients However appropriate combinations of two or more of the available secondary antituberculosis drugs usually can provide effective treatment The direct method can be used to test for bacterial susceptibility to selected concentrations of these secondary drugs as well

One of the earliest indications of a satisfactory chemotherapeutic response in a patient is the disappearance of acid fast bacilli from his excretions Sputum *conversion* usually precedes roentgenographic evidence of the regression of tuberculous lesions Frequent cultures i e at intervals of 2 to 4 weeks will provide the clinician with one of the best and most rapid qualitative measures of response to treatment In those rare in

stances in which relapse or failure does occur these detailed bacteriologic studies can provide a retrospective understanding of the factors involved and provide guidance for treatment

Properly used contemporary laboratory methods can provide an earlier definitive diagnosis more rapid selection of appropriate and adequate drug regimens and a resulting reduction in the total hospitalization period for patients with tuberculosis The money saved through decreased hospitalization greatly exceeds that used to upgrade the available bacteriology services to provide the new and rapid laboratory tests needed for the modern treatment of tuberculosis

Concentration, Stained Smear and Culture Morning sputum or a collection of sputa over a period of 24 hours can be liquefied enzymatically or by any of various agents (*sodium hydroxide sulfuric acid oxalic acid* trisodium phosphate n acetyl cysteine in sodium hydroxide) which are bactericidal for many contaminating microorganisms but less so for tubercle bacilli (Kubica *et al* 1963) The liquefied sputum is centrifuged the sediment is washed and then planted on appropriate media Some laboratories plant two different dilutions of the final sediment for two purposes (1) to avoid the toxic effect of some sputum sediments which is occasionally encountered in practice and (2) to control the size of the inoculum in direct drug susceptibility testing so as not to obscure a high proportion of drug susceptible members of the population by overgrowth of drug resistant mutants on medium containing drug

Animal Inoculation In some laboratories a portion of the material prepared for culture is inoculated into the groin of young guinea pigs The animals are tested with tuberculin at intervals of a few weeks and examined for tuberculosis when the reaction becomes positive The relative values of the cultural and the animal inoculation methods vary in different laboratories The cultural method is to be preferred under nearly all circumstances

The general technics described above for sputum examination are also applicable to the examination of gastric contents (in children or when the patient cannot raise sputum), pleural fluid urine (smegma bacilli frequently may be found in stained films of urine sediments) and spinal fluid The fibrin web which forms after brief incubation of spinal fluid may be transferred directly to a slide and examined and cultured Biopsy material may be frozen then macerated and subjected to the above procedures

Positive findings are proportional to the frequency of examinations One or two negative findings do not rule out tuberculosis or other mycobacterioses

Serologic Methods

Many attempts have been made to devise a serologic test to aid in the diagnosis of tuberculosis The most widely studied serologic methods have been complement fixation reactions Such serologic tests have given too high a percentage of false positive" reactions with human sera to warrant their routine clinical use

New types of serologic reactions have been devised recently which involve the phenomenon of adsorption of antigens of tubercle bacilli onto erythrocytes When red cells so sensitized are exposed to sera containing antibodies against the adsorbed antigens the red cells are agglutinated (Middlebrook and Dubos 1948 Boyden 1951) If complement is added to the mixture of sensitized red cells and specific antiserum the red cells are lysed (Middlebrook 1950b) A third hemagglutination method in which tuberculin (PPD) is linked to formalized erythrocytes by bis diazotized benzidine, has been described (Cole and Farrell 1955) Methods for eliminating a serum fraction which appears to be unrelated to tuberculous infection or disease by column chromatography in DEAE cellulose or by treatment of the serum with mercaptoethanol suggest that the incidence of false positive reactions may be reduced but the limits of diagnostic usefulness of such tests involving hemagglutination or hemolysis have not yet been defined (Turcotte *et al* 1963)

An agar gel double-diffusion precipitate test is currently under study for the diagnosis of tuberculosis (Parlett and Youmans, 1959)

Tuberculosis in Animals

The host range of tubercle bacilli is almost unlimited The mammalian types can infect wild animals in captivity especially primates and domestic animals including dogs and cats (Hawthorne *et al* 1957) Avian tubercle bacilli infect—besides fowl—swine and rarely cattle sheep and horses In aquaria fishes and turtles occasionally suffer from progressive tuberculosis caused by mycobacteria pathogenic for poikilothermic animals

Large numbers of cattle are infected with the bovine type of tubercle bacilli and many have progressive disease Tubercle bacilli are often present in the milk of infected cows even if tubercles are not demonstrable in the udder * Most infected cattle in the United States have no gross pulmonary tuberculosis but only infected lymph nodes generalized tuberculosis is not frequent The infection is diagnosed by the subcutaneous tuberculin test (local inflammatory reaction often associated with fever) The demonstration of tubercle bacilli in the milk is rarely used for diagnosis For the eradication of cattle tuberculosis in the United States herds are tuberculin tested periodically and the reactors are slaughtered The rate of infection in the United States was reduced from about 5 per cent in 1917 to 0 1 per cent in 1963 In other countries less expensive methods are used such as slaughtering grossly tuberculous cows and segregating the reactors

Tuberculosis in the chicken is most conspicuous in the liver but the spleen the intestines the lungs and other organs are also affected Avian tubercle bacilli may be present in hens eggs The infection can be diagnosed by injection of avian tuberculin into the wattle In the United States between 1925 and 1963 about 4 1 per cent of chicken flocks were estimated to be infected with avian tubercle bacilli

Pigs are infected usually with bovine or avian tubercle bacilli and rarely with the human type Infection results from ingestion of contaminated milk or material contaminated with feces of tuberculous fowl The infection in pigs is usually localized in lymph nodes of the alimentary canal

* In the milk of tuberculous women tubercle bacilli are very rarely found unless there are tubercles in the mammary glands and this is most uncommon

MYCOBACTERIUM ULCERANS AND MYCOBACTERIUM BALNEI

Mycobacterium ulcerans (MacCallum *et al* 1948) is the primary etiologic agent of a chronic or subacute type of ulceration involving both cutis and adjacent subcutaneous tissues on either the upper or the lower extremities of human beings Direct films of the exudate of such lesions reveal many acid fast rods separately in bundles or in short cords indistinguishable morphologically from mammalian tubercle bacilli Visceral lesions have not been observed The epidemiology of the disease is obscure the numbers of individuals affected are small they live in rural areas and show no suggestively significant geographic distribution Cases have been reported from Australia West Africa and Mexico

The bacilli can be cultivated on any of the usual media suitable for tubercle bacilli provided that the temperature of incubation is maintained within the limits of 25 to 35 C Their rate of multiplication approximates that of the bovine and the human types of tubercle bacilli They are pathogenic for rats mice and guinea pigs but progressive lesions occur only in those anatomic parts which normally have a temperature lower than 37° C such as the extremities the tip of the nose the tail and the testes Thu here is little doubt that the anatomic localization of lesions is attributable to the odd temperature requirements of this mycobacterium Of some interest from a bacteriologic as well as an epidemiologic standpoint is the fact that no other qualitative differences between this organism and the classic mammalian strains of tubercle bacilli have been observed either in serologic or in tuberculin tests

Mycobacterium balnei (Norden and Linell 1951) is another species of pathogenic mycobacteria which causes granulomatous lesions of the extremities and is unable to multiply at temperatures above 35° C Swimming pools appear to be the principal source of infection with these organisms

Aquariums and tropical fish tanks can also be the source Indeed *M balnei* is identical with *M marinum* first isolated and named by Aronson (1926) These organisms differ from *M ulcerans* in their much more rapid multiplication in vitro and in vivo (in the footpad of the mouse and in chick embryos) at 33° C and in the photochromogenic yellow color of their colonies

Heterologous immunity has been demonstrated in mice between *M ulcerans* and *M balnei* BCG also induces a high degree of protection against challenge infection with both species In contrast *M ulcerans* and *M balnei* appear to be much less effective in eliciting immunity to virulent bovine tubercle bacilli (Larson and Wicht, 1963)

JOHNE S DISEASE

This is a specific enteritis of cattle sheep and deer caused by acid fast bacilli called Johne s bacilli (*M paratuberculosis*) The disease has a long incubation period and runs a very chronic course characterized by intermittent diarrhea and progressive emaciation without fever In certain countries it causes serious economic loss in cattle It is not pathogenic for man

M paratuberculosis is a short thick rod which is acid fast and does not form spores On primary isolation it can be cultivated only on media containing an as yet unidentified substance or substances present in acid fast bacilli or in alcoholic extracts of certain plants Twort and Ingram 1912) Recently a crystalline growth factor for *M paratuberculosis* named mycobactin has been isolated from *M phlei* This substance effectively substitutes for extracts of *M phlei* as a growth factor for *M paratuberculosis* but the lag phase for growth of the latter on egg media to which mycobactin is added is still remarkably long (Rose and Snow 1955)

The lesions are characterized by gross thickening of the mucosa of the small intestine and enlargement of the mesenteric lymph nodes without ulceration The cellular reaction about the bacilli which may be intracellular or extracellular is diffuse not localized as in tuberculosis Lymphoid and epithelioid cells are present giant cells are rare caseation and calcification do not occur A skin test with johnin analogous to tuberculin, is used as an aid in the diagnosis of this disease

LEPROSY

Since it is characteristically present in leprous lesions *M leprae* (Hansen s bacillus) has been accepted as the etiologic agent of leprosy, although no cultures have been obtained that produce the disease in experimental animals Several strains of acid fast organisms have been cultivated from leprous lesions but those which appear to be especially significant have not been propagated in successive cultures Sera of patients with leprosy do not react specifically with the so-called *M leprae* cultures

Shepard has recently observed that *M leprae* can be isolated and cultivated regularly in the footpads of mice (1963) It has long been known that the sites that seem to be most severely involved in human leprosy are those of the coolest tissues The mouse footpad is uniquely cool, closer to the temperature of human skin and nasal mucous membrane than any other readily available tissue in the laboratory In footpads the generation time during the logarithmic growth phase was 12 to 13 days

In histologic sections leprosy bacilli are from 1 5 to 8 microns long and from 0 2 to 0 5 micron in diameter They are straight or slightly curved and often occur in globular masses known as globi and in groups arranged as parallel rods As a rule leprosy bacilli are stained uniformly red with carbol fuchsin but occasionally granules can be seen Lepromin somewhat analogous to tuberculin is the name given to a suspension of lepromatous tissue rich in bacilli Mitsuda used it in skin tests as early as 1916

The disease occurs in nodular (cutaneous) and neural (anesthetic) forms Both are often present in the same patient Either of these forms may be manifested by lepromatous tuberculoid or rarely intermediate types of lesions In the lepromatous type the lesions contain many bacilli the lepromin skin test is negative and the prognosis is poor In the tuberculoid type there are few bacilli in the lesions the lepromin test is positive at 24 to 48 hours and again at 2 to 4 weeks and the prognosis is good

The tuberculin test is not affected by the

presence or the absence of leprosy On the other hand patients with active tuberculosis often give a positive reaction to lepromin and BCG vaccination usually produces lepromin as well as tuberculin conversions It may be assumed from these observations that there is little or no immunologic cross reactivity between the proteins of *M tuberculosis* and *M leprae* but these two organisms possess some common antigens Indeed the sera of patients with lepromatous lesions usually contain large amounts of antibodies against one or more components of tuberculin which are adsorbable onto red cells in the hemagglutination test In nonlepromatous cases the positives are fewer and the titres lower in spite of the fact that such individuals have a better prognosis (Lowe 1955 Cochrane 1955)

It would appear that immunization of mice with heat killed human tubercle bacilli or with BCG vaccine effects a definite but partial degree of suppression of multiplication of *M leprae* in the footpad (Shepard 1963) BCG is being studied as a possible agent for immunization against leprosy

In nodular leprosy the skin is raised over firm nodules (lepromata) which are most frequently present on exposed parts such as the face and the extremities The skin often ulcerates and secondary infections may set in The neural form affects peripheral particularly sensory nerves producing anesthesia Due to lack of sensation the affected hands and feet are likely to be injured leading to mutilation of these parts Lesions may develop in almost all organs except voluntary muscle The cartilage of the nose is often destroyed the mucous membranes of the mouth and the nose the eye and the testes are frequently affected Lesions are common in lymph nodes They are characterized by granulation tissue well supplied with blood vessels and lymph ducts There are many large mononuclear cells containing numerous acid fast bacilli and fat globules Multinucleated giant cells are often present Most of the acid fast bacilli are in the endothelial cells of the lymph ducts and the blood vessels In the neural lesions the bacilli are in the perineural and the epineural tissue producing extensive formation of granulation tissue The nerve fibers degenerate with loss of motor function as well as sensation Therefore the hands and the feet may undergo atrophy

The disease u ually progresses very slowly the neural type is particularly insidious Remissions often occur this fact has made evaluation of possible therapeutic measures very difficult Chaulmoogra oil and its derivatives have been used for many decades in the treatment of leprosy but not until the advent of the sulfonamidelike compounds have unequivocal chemotherapeutic effects been demonstrable The sulfones are the drugs of choice in the treatment of certain forms of leprosy (Bushby 1958) Isoniazid is ineffective in dosage of 3 to 5 mg/Kg/day However in footpads of mice isoniazid and para aminosalicylic acid in adequate dosage suppressed bacillary multiplication completely (Shepard 1963) This combination of drugs has not yet been given adequate clinical trial in leprosy

The bacteriologic diagnosis is most often made by finding acid fast bacilli in scrapings of the nasal mucosa and in the tissue fluid expressed after superficial incisions of the skin in certain areas There is no serologic test of value It may be noted that a large percentage of leprous patients who have neither syphilis nor yaws are Wassermann positive

The disease is believed to have originated in Central Africa whence it spread to all parts of Europe and subsequently to the rest of the world It is widely disseminated in the Orient It is estimated that there are 3 million lepers in the world about 750 in the United States and over 30 000 in Central and South America

All human races are susceptible to the disease and it is said that susceptibility has a genetic basis This opinion is based on the greater incidence of the disease in siblings than in their parents in the same household However this conclusion may not be valid susceptibility is greatest in childhood The ratio of the incidence of the disease in males to the incidence in females is approximately 2 to 1

The incubation period varies from a few months to many years most commonly from 2 to 4 years The ulcers of the skin and the nasal discharge are probably the most important sources of infection The disease is not highly contagious and probably *is* ac-

quired by infecting superficial abrasions of the skin It is likely that persons can acquire the infection without developing manifest disease It has not been shown that animal vectors spread the infection The most effective prevention in endemic areas is the segregation of patients in leprosaria while patients in temperate climes need not be segregated The removal of children from leprous parents has proved to be a useful prophylactic measure

Unlike the tubercle bacillus *M leprae* of man does not produce progressive infection in animals But rats in many countries have a disease called rat leprosy which is characterized by nodular skin lesions containing acid fast bacilli (*M leprae murium*) difficult to cultivate and nonpathogenic for other species of animals There is no evidence that rat leprosy can be transmitted to man or that the rat plays any role as vector for human leprosy

It is interesting to compare *M tuberculosis* and *M leprae* Tubercle bacilli have many types and their host range is almost unlimited they can be cultivated on artificial media There are only 2 known types of *M leprae* and they are exclusively pathogenic either for man or for rats mice and hamsters *M leprae* has not been *cultivated* in vitro (Hanks and Gray 1956) Tuberculosis has been the disease of urban civilization and occurs in domestic animals Leprosy is prevalent in the tropics among people who live under rural conditions After its introduction to Europe from the Near East the disease became endemic until the 16th century and then disappeared

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22

The Anthrax Bacillus

Anthrax is primarily an acute infectious disease of domestic herbivorous animals The disease in man is contracted by contact with infected animals or contaminated animal products *Bacillus anthracis*, the causative agent is a large gram positive rod shaped organism which forms resistant spores The organism is heavily encapsulated in vivo and under appropriate cultural conditions in vitro

HISTORY

The disease has been known from antiquity and satisfactory descriptions of the typical lesions in animals and man have been available for about 200 years *B anthracis* was the first microorganism to be established as the etiologic agent of a disease It was discovered in the blood of infected sheep by Rayer in 1850 and experimental transmission of the disease was demonstrated by Davaine in 1863 Koch (1877) described the isolation and the cultivation of the organism the formation of spores the production of the disease in experimental animals and the recovery of the organism from the experimental infection These classic experiments represented a major advance in the understanding of anthrax and provided the basic concepts and methods for the rapid expansion of medical bacteriology Immunization of animals with attenuated living vaccines was introduced by Pasteur and a field trial at Pouilly le Fort in 1881 established the effectiveness of the procedure The subsequent introduction of the vaccines into general use provided a major stimulus for early investigations in immunology

MORPHOLOGY AND CULTURAL CHARACTERISTICS

Bacillus anthracis is a large gram positive, spore forming rod 1 to 1 5 microns in width and 4 to 8 microns in length Large capsules are formed in vivo and under appropriate cultural conditions in vitro Spores are not formed in the living animal In smears from an infected host the organisms lie singly or in short chains capsules are formed and may be demonstrated by Giemsa or similar methods of staining The appearance of organisms in artificial media is considerably different In young cultures on nutrient agar the bacilli are nonencapsulated rods with square ends arranged in strands of long chains Under aerobic conditions sporulation begins toward the end of logarithmic growth and is usually well advanced after 48 hours of incubation Spores are oval in shape and are formed equatorially They are liberated by autolysis of the vegetative cells pairs or short chains of spores may remain connected by linkages which are evidently extensions of the spore coat (Roth *et al* 1956) The organism is nonmotile strains described as motile probably were erroneously identified (Sterne and Proom 1957)

Typical colonies of virulent, wild type strains that have grown 24 hours on a medium such as nutrient agar are flat, dull

gray and medusa head in appearance The last characteristic is caused by outgrowths which radiate from the margin of the colony and then curve back toward it so that the colony appears to be spinning The organism is nonhemolytic or only slightly hemolytic on blood agar and this characteristic serves to differentiate it from many saprophytic bacilli When grown in the presence of carbon dioxide the organisms are encapsulated and produce colonies that are round and mucoid (Thorne 1960)

The organism grows well on nutrient agar and other general media In chemically defined media the requirements for rapid growth are rather complex and include thiamine and a considerable number of amino acids in addition to inorganic salts (Proom and Knight 1955) Complex interrelationships with respect to amino acid requirements now recognized in many organisms were first demonstrated with *B anthracis* (Gladstone 1939) Purines and pyrimidines stimulate growth of many strains Although molecular oxygen is necessary for sporulation germination of spores occurs under anaerobic as well as aerobic conditions (Roth and Lively 1956) Rapid germination of spores occurs in the presence of adenosine L alanine and L tyrosine (Hills 1950) The organism grows best aerobically but growth also occurs under strict anaerobic conditions Acid without gas is produced in the presence of glucose levulose sucrose maltose trehalose and dextrin Mannose and the pentoses are not fermented The organism produces a typical heterolactic fermentation of glucose acetic succinic lactic and formic are the principal acids formed (Puziss and Rittenberg 1957)



FIG 1 Typical lesion of cutaneous anthrax 3 to 4 days old The black center is surrounded by a ring of vesicles painless edema encircles the lesion (Gold 1955)



FIG 2 Colony of virulent *B anthracis* grown on nutrient agar and stained with methylene blue (A) The whole colony × 45 (B D) Border of the same colony (× 145 × 400 and × 1 600 respectively) Note parallel arrangement of the bacterial filaments (Stein 1947)



FIG 3 Colonies of virulent *B anthracis and of an avirulent nonencapsulated mu tant* grown on bicarbonate containing medium in the presence of carbon diox ide The round mucoid colonies of the virulent strain are readily distinguished from the irregular white colonies of the mutant (K L Burdon)

The fermentation is not unlike that carried out by saprophytic members of the genus *Acetylmethylcarbinol glycerol and* 2 3 bu tylene glycol are produced nitrates are re duced to nitrites and gelatin is slowly lique fied Protease amylase catalase lecithinase collagenase and D- and L-amino acid trans aminase may be demonstrated in whole cells homogenates or culture filtrates Certain strains become susceptible to the action of lysozyme when grown in the presence of carbon dioxide (Gladstone and Johnston 1955)

VARIATION

The foregoing description is applicable to typical virulent strains as isolated from the field Variation occurs among wild type strains in characteristics such as virulence nutritional requirements and sensitivity to antibiotics bacteriophage and lysozyme In addition, mutants are obtained readily under appropriate conditions of cultivation in the laboratory Serial transfer in laboratory media particularly at elevated temperature *leads to* a *gradual* decrease in virulence Use of strains attenuated in this manner as living vaccines was introduced by Pasteur The colonies of the attenuated vaccine strains are smoother and more dome shaped than are those of virulent strains and the char acteristic marginal projections are absent The attenuated strains have acquired the ability to form capsular material when grown in air on nutrient agar Overgrowth of viru lent cells by avirulent mutants occurs slowly and attenuation is a gradual process (Preisz 1911) Nonsporulating mutants may appear particularly during cultivation in the pres ence of relatively high concentrations of cal cium salts which inhibit sporulation and favor the emergence of nonsporulating mu tants (Renaux 1952) Nonsporulating strains may be virulent or avirulent in dicating that sporulation and virulence are *independent characteristics Transduction of* sporogenesis in recently isolated nonsporing strains has been described (Stamatin 1959)

The colonies of virulent strains are rough when grown on nutrient agar and the rela tively smooth growth of the attenuated strains has led to the misconception that *B anthracis* represents an exception to the rule that loss of virulence accompanies the S→R variation However the fully virulent organisms *form large capsules in vivo* and also during growth in vitro in the presence of carbon dioxide Under the latter condi tions the colonies are mucoid in appearance indicating that the fully virulent strains are not rough if cultivated under appropriate nutritional conditions After about 48 hours of incubation small rough outgrowths ap pear on the margin of the colonies and careful selection from these areas yields avirulent mutants that are rough under all conditions of cultivation and have lost the ability to synthesize the capsular polypep tide (Sterne 1937 Thorne 1960) Nonen capsulated mutants also arise in vivo and may represent the predominant form in animals that survive for a sufficient time after infection (Beck *et al* 1960) Although encapsulation is essential in the initial es

tablishment of infection it evidently is unnecessary and is selected against after certain defense mechanisms of the host have been overcome Reversion of nonencapsulated strains to the encapsulated virulent form occurs with detectable frequency in about half the strains studied (Meynell 1963) Stable nonencapsulated mutants have come into general use as viable vaccines for immunization of animals

Additional colonial variants that arise in old cultures include avirulent mucoid mutants that are heavily encapsulated when grown on nutrient agar in air several types of smooth colonies and phantom colonies that grow as a thin film on the surface of the medium (Nungester 1929) Nonproteolytic mutants occur with appreciable frequency in a variety of cultures loss of the protease has no effect on virulence (Wright *et al* 1962)

ISOLATION AND IDENTIFICATION

Isolation of *B anthracis* presents no problem if the specimen contains an appreciable number of the organisms and is fresh and not excessively contaminated Nutrient agar blood agar and egg yolk agar have been recommended for primary isolation Blood agar has the advantage that many colonies otherwise resembling those of *B anthracis* may be excluded by their strong hemolytic activity Typical colonies may be selected and identified by cultural methods and virulence tests The organism is rapidly killed by putrefactive processes and cultural methods may yield negative results with specimens from decomposed carcasses Inoculation of mice or guinea pigs may allow primary isolation when cultural methods are unsuccessful but contaminating organisms particularly clostridia may cause rapid death of the animals In these cases superficial inoculation by application of the suspension to the scarified skin may prove to be successful Isolation of spores from heavily contaminated samples such as soil manure bone or hair may be more difficult, and methods must be selected for each specimen Best results in isolation from goat hair and related samples are obtained by suspension of the material in a detergent solution treatment at 70 C concentration by centrifugation and inoculation of mice (Biegeleisen *et al* 1961) Media containing selective inhibitors for the more troublesome contaminating organisms may facilitate primary cultural isolation of *B anthracis* (Pearce and Powell 1951 Morris 1955 Gillessen and Scholz 1961)

Identification of *B anthracis* involves primarily differentiation from other members of the genus Bacillus and particularly from *B cereus* For tentative identification reliance may be placed on colonial and microscopic morphology the virtual absence of hemolytic activity on sheep blood agar and the absence of motility Supporting evidence may consist of sensitivity to 10 units/ml of penicillin ability to form capsules on bicarbonate agar in the presence of 20 per cent carbon dioxide but not on nutrient agar in air and susceptibility to bacteriophage Although motility is sufficient to disqualify a culture as *B anthracis* the absence of motility is only indicative because nonmotile strains of *B cereus* although uncommon have been described (Ivanovics and Foldes 1958) Most investigators have concluded that susceptibility to the gamma phage of Brown and Cherry (1955) represents a highly specific test for *B anthracis* However recent studies with gamma and other phages indicate that no one phage is capable of lysing all strains of *B anthracis* (Buck *et al* 1963) Further study will be required to establish the significance of negative phage susceptibility tests

Final identification of virulent *B anthracis* is usually based on demonstration of pathogenicity for mice or guinea pigs with appearance of encapsulated organisms in the blood and the tissues at death Small inocula should be used for pathogenicity tests because saprophytic members of the genus may produce fatal infection or intoxication if inoculated in large numbers especially intraperitoneally The fluorescent antibody technic using appropriate antisera appears to be useful for detection and tentative identification of *B anthracis* in tissue sections and smears (Cherry and Freeman 1959) Strains possessing little or no pathogenicity but typical of *B anthracis* in other respects may represent attenuated or avirulent mutants Slow or negligible fermentation of salicin slow reduction of methylene blue and slow liquefaction of gelatin are addi

tional characteristics of *B anthracis* that may aid in identification (Stein 1944) Contamination of cultures with *B cereus* represents a potential basis for confusion in identification of isolates in evalution of methods of identification and in study of taxonomic relationships (Burdon and Wende 1960)

PATHOGENICITY

Virtually all animals are susceptible in some degree to infection with *B anthracis* In nature or under the conditions of normal animal husbandry infections have occurred in cattle sheep horses goats buffaloes water buffaloes minks swine deer ostriches and elephants Outbreaks occur occasionally in zoos and many species of carnivorous animals and birds have been infected under these circumstances by ingestion of contaminated meat (Stein and Van Ness 1955) Experimental infections have been produced in mice rats guinea pigs rabbits and many wild rodents monkeys chimpanzees and even frogs and fish Susceptibility varies greatly among different species and strains Of the common laboratory animals mice and guinea pigs are the most susceptible to challenge by the cutaneous route (LD_{50} less than 5 spores) Rabbits monkeys goats and sheep are also highly susceptible White rats cats dogs cattle and swine are much less susceptible and usually survive subcutaneous injection of 10^6 spores although a localized infection may occur at the site of inoculation The LD_{50} of anthrax spores for the white rat was found to increase by a factor of 10^5 during the first 5 weeks of the life of the animal germ free animals remained highly susceptible at 5 weeks of age (Taylor *et al* 1961) The susceptibility of a species may be altered experimentally by changes in body temperature by administration of certain hormones or by preexisting infection with unrelated organisms

Cutaneous anthrax or malignant pustule is the most common form of the disease in man Frequently the infection develops at the site of a scratch or a minor abrasion The organism is apparently unable to penetrate the intact skin Cutaneous lesions occur almost exclusively on exposed portions of the body more than 95 per cent occur on the head the neck and the upper extremity The evolution of the cutaneous lesion is described in the section on diagnosis Before the advent of antibiotic therapy the mortality of cutaneous anthrax over a 20 year period in the United States was 21 per cent of the 1 683 reported cases (Smyth 1941) However the true mortality of untreated human anthrax is difficult to estimate because failure to report less severe cases tends to raise the apparent mortality and therapeutic measures doubtless reduce the mortality It is evident that in the absence of therapy an appreciable proportion of cutaneous infections in man would progress to generalized infection and death

Infection may occur not only by the cutaneous route but also via the respiratory and the alimentary tracts and experimentally via the intramuscular the intraperitoneal and the intravenous routes of inoculation Although there is no evidence that the respiratory route is of importance in pathogenesis of the natural disease in animals experimental inhalation anthrax has received considerable study since the original investigations of Buchner in 1888 In the guinea pig spores deposited in the alveoli are taken up by alveolar phagocytes and carried to the tracheobronchial lymph glands Here the bacilli that escape destruction by the normal defensive mechanisms of the host multiply rapidly the infection spreads via the efferent lymphatics to the circulation (Ross 1957) The respiratory LD_{50} of anthrax spores for susceptible animals such as guinea pigs and monkeys is approximately 20 000 organisms the size of the dose suggests that the clearing mechanisms of the lung are usually effective and only the occasional spore is able to initiate progressive infection Many of the spores deposited in the lung have been found to remain inactive for long periods Viable spores can be detected in the lungs of immunized monkeys 100 days after respiratory challenge (Henderson *et al* 1956)

The infectivity of aerosols for guinea pigs and monkeys is influenced markedly by particle size The LD_{50} increases markedly as the particle size is increased from 1 to 12 microns (Druett *et al* 1953) Particles less than 5 microns in diameter are much more

likely to penetrate into the alveoli and be deposited on the alveolar wall (Harper and Morton 1953) Infectivity of the aerosol may be increased by addition of certain detergents and other substances to the spore suspension prior to aerosolization (Young and Zelle 1946 Barnes 1947)

Inhalation anthrax of man (woolsorter's disease) was formerly relatively common Improvements in working conditions in industries that deal with contaminated hair and hides have reduced the exposure of workers to spore bearing dust and presumably for this reason the incidence of inhalation anthrax has declined However exposure to airborne spores has not been eliminated and the disease has not disappeared Scattered cases are diagnosed periodically and an outbreak of 5 cases with 4 deaths occurred in 1957 among employees of a textile factory in New Hampshire (Plotkin *et al* 1960 Brachman *et al* 1961) The mortality of diagnosed cases is 80 per cent or more although it is possible that nonfatal cases escape diagnosis Occurrence of subclinical infections in occupationally exposed textile workers has been postulated on the basis of serologic studies (Norman *et al* 1960) Development of the disease in man resembles that of the experimental disease in the guinea pig The principal lesions are acute mediastinitis septicemia and widespread toxic effects referable to septicemia Pneumonic lesions are usually minimal or absent (Albrink 1961)

Fatal infection of cattle and severe infection of swine may be produced by the oral route although these animals are resistant to cutaneous challenge (Schlingman *et al* 1956) Dogs sheep and many other animals may be infected by feeding however guinea pigs and rabbits are not infected by oral administration of 10^9 spores (Druett *et al* 1953) It is evident that susceptibility by different routes of infection may vary independently and that the infective dose by one route of challenge gives no indication of susceptibility by another route Human infection by the oral route is of negligible importance in civilized countries but significant outbreaks with high mortality occur in primitive societies in which meat from infected animals is used for human food Additional information is needed in regard to the infective dose and mortality in such outbreaks Meningitis due to *B anthracis* is a relatively rare and almost invariably fatal manifestation of human anthrax and is usually a complication of primary infection elsewhere (Haight, 1952)

No direct information is available in regard to the infective dose for man by the various routes and estimates of human susceptibility must be inferred from indirect evidence The significant mortality of cutaneous anthrax indicates that man is more susceptible than white rats cattle and swine The tissue reaction to infection and the occurrence of spontaneous recovery from cutaneous infection suggest that man is less susceptible than the guinea pig the rabbit and the monkey Susceptibility by the respiratory route is even more difficult to estimate There is no evidence that man is less susceptible to small particle aerosols than are the guinea pig and the monkey

FACTORS IN INFECTION AND RESISTANCE

Anthrax has occupied an important position as a model infection for investigation of mechanisms of infection and resistance This has resulted from the acute course of the infection with its dramatic terminal bacteremia the wide variation in the susceptibility of different species and the economic and medical importance of the disease Early investigations have been reviewed by Sobernheim (1931) and Eurich and Hewlett (1930) and more recent work by Smith and Keppie (1955) and by Sterne (1959) Organisms injected into highly susceptible animals become encapsulated and proliferate freely Few leukocytes accumulate and rapid generalization of the infection occurs In less susceptible animals the course of the infection is similar for the first few hours After this period however large numbers of leukocytes appear in the lesion and the organisms lose their capsules and disintegrate without conspicuous phagocytosis (Cromartie *et al* 1947) During growth in vivo the organism produces aggressins which act to overcome the normal defensive mechanisms of the host. These substances inhibit the bactericidal or phagocytic activities of the host and permit progressive in

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fection Fractionation of infected tissue or of organisms grown in vivo reveals 3 substances that appear to be capable of contributing to in vivo aggressin activity the glutamyl polypeptide of the capsule the anthrax toxin and an intracellular anticomplementary protein The glutamyl polypeptide evidently acts by protecting the organism against opsonization by normal serum, the toxin by direct harmful effect on phagocytes The significance of the intracellular protein has not been established (Keppie *et al* 1963) The bactericidal activity of serum leukocytes and tissue extracts is evidently due to the presence of basic polypeptides containing lysine The polypeptide causes a rapid inhibition of oxygen uptake in vitro by suspensions of *B anthracis* presumably its protective activity in vivo is exerted in a similar manner (Bloom and Prigmore 1952) The course of the infection is influenced by the relative abilities of the organism to elaborate aggressins and of the host to mobilize bactericidal substances The encapsulated bacilli are resistant to the phagocytic cells of relatively resistant as well as susceptible animals and it is probable that phagocytosis is of minor importance in natural immunity to infection with virulent strains

The mechanism of death in anthrax has aroused the interest of investigators for many years Sterile extracts of infected tissue have edema producing activity in the skin of normal animals but lethal activity cannot be demonstrated Culture filtrates organisms grown in vitro or in vivo and killed in various ways and extracts of such organisms likewise exhibit no significant toxic activity It seemed to be possible that the massive terminal bacteremia that occurs in the guinea pig and certain other species is directly responsible for death Blockage of capillaries or exhaustion of oxygen or of essential host nutrients have been suggested as basic mechanisms of death in the infection These theories were rendered untenable and the problem was greatly clarified by the investigations of Smith Keppie and associates (see Smith and Keppie 1955) Treatment of infected guinea pigs with streptomycin promptly terminates the infection even when the drug is administered a few hours before death would otherwise have occurred Multiplication of the organisms ceases and the bacteremia rapidly disappears However when the bacteremia exceeds 3 x 10^6 chains of organisms per ml of blood the animals die of anthrax in spite of complete clearance of organisms from the circulation The critical stage is reached at a point only 1/300th of the level of the bacteremia at death in untreated animals These experiments demonstrate that a factor other than the physical presence of the organisms in the circulation is of critical importance and have again directed attention to other mechanisms and to search for a lethal toxin

Detailed study of infected guinea pigs revealed that, in the terminal phases the animals present a classic syndrome of secondary shock The manifestations include reduced blood volume and pressure hemoconcentration the presence of edema and hemorrhage and fall in body temperature Disturbances in electrolyte balance and in phosphate and carbohydrate metabolism are prominent and there is evidence of acute renal failure A renewed search for the factor responsible for these effects led to the finding that sterile heparinized plasma of guinea pigs dying or near death from anthrax is capable of producing extensive edema in the skin of normal guinea pigs and is lethal to mice and guinea pigs when injected intravenously or intraperitoneally The toxicity is specifically neutralized by anthrax antiserum Presumably the plasma toxin is closely related to the tissue damaging factor detected in extracts of anthrax lesions

Initially the plasma toxin was separated by ultracentrifugation into 2 fractions both of which were necessary for activity, and one of which Factor II could be replaced by protective antigen produced in vitro (Smith *et al* 1956a) The other component Factor I was subsequently produced in *vitro and purified by chromatographic* methods (Thorne *et al* 1960 Sargeant *et al* 1960) Factor I is a protein with a strong chelating action for metal ions it forms a specific line of precipitation with appropriate anthrax antisera in Ouchterlony plates When combined with Factor II it produces edema in the rabbit skin although the lethal activity in mice and rats is low, relative to the edema producing activity

Factor III or rat lethal factor has been detected and partially purified this factor in combination with protective antigen is evidently responsible for the lethality of plasma toxin (Stanley and Smith 1961 Beall *et al* 1962) Negative results have thus far been obtained in attempts to demonstrate enzymatic activity in a mixture of the three purified factors of the toxin and the biochemical basis of the toxicity remains unknown (Stanley and Smith 1963) Intravenous injection of crude toxin leads to liberation into the blood of alkaline phosphatase However this effect is produced by factors related to phospholipase and not by the anthrax toxin (Slein and Logan 1962)

ANTIGENIC STRUCTURE

Three antigens of the organism have been recognized for many years and extensively studied the protective antigen (Factor II of the anthrax toxin) the capsular polypeptide and the somatic polysaccharide In addition Factors I and III of the toxin are evidently antigenic proteins (Stanley and Smith 1961 Beall *et al* 1962) Protective antigen is the substance primarily responsible for development of immunity to infection in most animals Bail in 1904 demonstrated this antigen in edema fluid of anthrax lesions and these observations have been confirmed and extended by subsequent investigators Attempts to produce protective antigen in vitro were unsuccessful until Gladstone (1946) demonstrated elaboration of the antigen during growth of the organism in whole serum under critical conditions Subsequently Wright *et al* (1954) obtained elaboration of protective antigen in chemically defined nonprotein media and these observations have been confirmed and extended (Belton and Strange 1954 Thorne and Belton 1957 Wright *et al* 1962) The antigen is a protein which is elaborated into the culture medium only under particular cultural conditions one of the most consistent nutritional requirements is for bicarbonate ion Bicarbonate evidently alters the permeability of the bacterial cell so that protective antigen is elaborated into the medium in its absence antigen accumulates intracellularly (Puziss and Howard, 1963)

Protective antigen can be detected and measured by means of its immunizing activity in rabbits or guinea pigs Recently more precise and convenient methods for estimation of protective antigen have been proposed The role of protective antigen in the anthrax toxin discussed above provides the basis for a procedure for its estimation in vivo (Belton and Henderson 1956) The antigen fixes complement in the presence of appropriate antisera and titrations utilizing this property correlate significantly with activity in immunization of guinea pigs (McGann *et al* 1961) Even more useful however has been the association of protective antigen with a line of precipitation demonstrable by the method of Ouchterlony (Thorne and Belton 1957) This association has provided a basis for detection and estimation of the antigen and its antibody and for study of their biologic activities

The principal component of the capsule is a polypeptide of glutamic acid This substance plays a major role in virulence of the organism since nonencapsulated mutants which do not elaborate polypeptide are avirulent The polypeptide has aggressin activity and because of its concentration around the organism it probably exerts a considerable effect in preventing phagocytosis (Smith and Keppie 1955) Antisera reactive with the isolated polypeptide in precipitin and complement fixation reactions may be prepared by immunizing animals with large numbers of encapsulated organisms However recent studies indicate that the reactive substance in such antisera is not antibody but rather a basic protein similar to serum lysozyme Thus the antigenicity of this polypeptide must be considered uncertain pending further study (Leonard and Thorne 1961)

Polypeptides similar chemically to that of *B anthracis* are elaborated by *B subtilis* and by certain other *Bacillus* species The *B anthracis* polypeptide is composed of D-glutamic acid in gamma linkage (Thorne 1960) whereas the polypeptide elaborated by *B subtilis* contains also L glutamic acid the proportion varying with cultural conditions

The somatic polysaccharide contains equimolar quantities of D-glucosamine D-galactose and acetic acid (Ivanovics 1940a) A small peptide moiety containing α-ϵ-diami

nopimelic acid is closely associated with the polysaccharide and the complex appears to form part of the cell wall (Smith *et al*, 1956b) The polysaccharide is present in all strains of *B anthracis* thus far studied and also in some strains of *B cereus* (Ivánovics and Foldes 1958) The polysaccharide reacts in high dilution with antianthrax serum and cross reacts with the polysaccharide of type 14 pneumococcus and with partially hydrolyzed blood group A substance (Iván ovics 1940b) The polysaccharide is devoid of aggressin activity and evidently plays no important role in virulence Polyglycero phosphate an antigen common to many gram positive organisms has been detected in extracts of *B anthracis* (McCarty 1959) Immunoelectrophoretic studies of culture fil trates and cell homogenates reveal numerous uncharacterized antigens

ACQUIRED RESISTANCE AND IMMUNIZATION

The observation that animals that survive anthrax become resistant to reinfection has been amply confirmed since the initial report of Chauveau in 1880 Second attacks of human anthrax are rare but apparently au thentic instances have been reported Vac cines composed of suspensions of killed or ganisms produce no significant increase in resistance Pasteur introduced the use of attenuated strains for vaccination of domes tic animals in 1881 and this method with subsequent modifications and improvements has been widely used where anthrax is a significant problem Originally the organism was attenuated by growth at 42° to 43° C and 2 vaccines of different degrees of at tenuation were used the first was virulent for mice but avirulent for guinea pigs and rabbits and the second was virulent for guinea pigs and some rabbits The use of 2 vaccines was gradually discarded and a sin gle vaccine virulent for guinea pigs but not for rabbits came into general use Saponin is usually added and in proper concentration it increases the antigenicity of the prepa ration

In practice it has proved to be difficult to maintain the virulence of the Pasteurian vac cines at the proper level If the vaccine is too virulent excessive losses occur among vaccinated animals If the virulence is in sufficient the vaccine is unable to produce the degree of infection that is necessary for development of immunity This difficulty has been overcome by the introduction of nonencapsulated spore vaccines by Sterne (1939) The nonencapsulated mutants have lost the ability to form the glutamyl polypeptide and are essentially avirulent They sporulate well and vaccines are read ily prepared on a large scale These vaccines produce smaller losses among vaccinated animals than do Pasteurian vaccines and a single preparation is suitable for all species of animals They were first introduced and used on a large scale in South Africa and have largely replaced the Pasteurian vac cines elsewhere None of the living spore vaccines is generally considered safe for im munization of man although large scale trials in man of vaccines of the Stern type have been reported from the USSR The vaccines have been administered by the res piratory as well as by the cutaneous route apparently no significant untoward reactions were encountered (Aleksandrov *et al* 1961)

For many years attempts have been made to develop effective nonviable antigens but until recently none of the preparations has afforded significant immunity to infection The development by Gladstone and subse quent investigators of conditions for elabo ration of protective antigen in vitro has been described above This antigen combined with alum or aluminum hydroxide is highly effective in immunization of guinea pigs rabbits monkeys sheep and cattle (Schling man *et al* 1956 Darlow *et al* 1956 Puziss and Wright 1963) The antigen is well tol erated in man and when it was tested in occupationally exposed textile workers it reduced the incidence of anthrax more than 90 per cent (Brachman *et al* 1962)

DIAGNOSIS

Prompt and accurate diagnosis of cuta neous anthrax in man usually presents no serious difficulty The characteristic lesion begins as a small red macule which enlarges and local edema of varying extent gradually develops in the surrounding tissue A vesicle filled with clear fluid occupies a central

position and is soon followed by satellite vesicles The organism may be easily demonstrated in the early lesion by cultural methods and frequently may be detected in stained smears Fluid and scrapings from the base of a previously unopened vesicle should be used for preparation of slides and of cultures on nutrient or blood agar Microscopic examination of Gram or Giemsa stained films will frequently reveal the organism and permit a rapid tentative diagnosis The lesion gradually becomes necrotic with evolution of the black eschar characteristic of anthrax At this stage demonstration of the organism is less certain and secondary infection may complicate the bacteriologic diagnosis Occasionally inoculation of mice or guinea pigs will allow isolation of the organism when direct cultural methods fail Antibiotic therapy usually causes rapid disappearance of the organism even though evolution of the local lesion continues Therefore specimens for bacteriologic study should be obtained before therapy is initiated

Extension to the regional lymph nodes and to the blood occurs in progressive infections blood cultures may be positive in advanced infections and in untreated cases are invariably positive at death The cutaneous lesion is not painful but tenderness of the regional lymph nodes does occur A history of occupational exposure to anthrax is of great assistance in the diagnosis particularly in areas such as the United States where the disease is uncommon Conventional serologic procedures have been of little value in diagnosis

Diagnosis of respiratory anthrax is a much more difficult problem because of the mild and nonspecific nature of the early symptoms and the fulminating course of the advanced disease which may resemble cardiac failure or cerebrovascular accident Mediastinal widening demonstrable by roentgenography is suggestive of the disease and gram stained smears of blood sputum or cerebrospinal fluid may reveal the organism (Plotkin *et al* 1960) Textbooks of veterinary pathology may be consulted for descriptions and diagnostic procedures applicable to the disease in animals (Hutyra *et al* 1946 Sterne 1959) The Ascoli thermoprecipitin reaction is of value in postmortem diagnosis Infected tissue is ground in saline heated for 5 minutes and filtered and the filtrate is layered over appropriate antianthrax serum In positive reactions a ring of precipitate is formed at the interface between the two fluids

THERAPY

Antisera prepared by hyperimmunization of animals with living cultures were used for many years in treatment of anthrax It seems that such antisera alone or in combination with neoarsphenamine produced a considerable reduction in the mortality of cutaneous anthrax With the advent of the sulfonamides a further reduction in mortality was obtained These drugs in their turn have been superseded by the antibiotics and the mortality of promptly diagnosed and adequately treated cases of cutaneous anthrax is now essentially zero The anthrax bacillus is susceptible to penicillin streptomycin tetracyclines and erythromycin but many strains are relatively resistant to chloramphenicol (Plotkin and Brachman 1964) Experimental infections in animals and many cases of human cutaneous anthrax have been treated satisfactorily with penicillin and this drug is generally considered to be most effective Tetracyclines and erythromycin are also effective and may be preferred under some circumstances (Gold 1955) Emergence of antibiotic resistance has not been a significant problem in therapy although virulent antibiotic resistant strains may be obtained by selection in the laboratory Antibiotics rapidly halt the extension of the disease and sterilize the tissues but they do not reverse the toxic processes initiated by the infection The primary lesion usually progresses to formation of the typical eschar despite early therapy The death of infected guinea pigs that had received doses of streptomycin sufficient to destroy all viable organisms has been discussed previously

Little information is available regarding antibiotic therapy of respiratory anthrax in man The early symptoms are mild and nonspecific and the existence of acute disease is seldom recognized until terminal symptoms appear Treatment has usually been initiated late in the disease and the unfavor

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Prompt and accurate diagnosis of cutaneous anthrax in man usually presents no serious difficulty The characteristic lesion begins as a small red macule which enlarges and local edema of varying extent gradually develops in the surrounding tissue A vesicle filled with clear fluid occupies a central

CONTROL MEASURES

Major emphasis in the control of human anthrax is placed on minimizing contact of man with infected animals or contaminated animal products The world wide problem is complex and the most appropriate measures vary in different areas and with different groups Economic and sociologic factors are interwoven with the purely medical and veterinary aspects of the problem Obviously the most satisfactory measure would be the elimination of the disease in animals Modern methods of immunization are capable of producing a marked reduction in incidence The organisms have been shown to remain viable in soil for many years and many agricultural areas are heavily contaminated The extent to which the disease can be eliminated by an effective program of immunization will doubtless vary with soil and climatic conditions Mass immunization has virtually eliminated the disease in South Africa (Sterne 1959) In colder countries in which the disease is sporadic outbreaks of animal anthrax have frequently been traced to imported foodstuffs particularly bone meal This material may be rendered safe by proper steam treatment during processing

When outbreaks of animal anthrax occur prompt diagnosis isolation of sick animals and suitable disposal of the carcasses are essential to limit the spread of the disease Prompt vaccination of healthy animals on the premises may also be indicated (Stein 1947) It is axiomatic that when anthrax is suspected autopsies should not be performed in the field so that further contamination of the area and sporulation of the organisms in the carcass may be avoided Disposal of carcasses by complete cremation or deep burial is imperative Proper disinfection of the surroundings represents a difficult problem since most of the disinfectants that are effective with vegetative organisms are without action on anthrax spores Strong hypochlorite solutions are the reagents of choice

Measures for the control of industrial anthrax include disinfection of animal products such as hides and hair that originate in areas in which the disease is widespread These materials may be heavily contaminated and economically feasible methods of disinfection that may be applied routinely without damage to the material are not easy to devise Observations on sterilization by heat have been reviewed by Schneiter and Kolb (1948) Washing with soap and exposure to warm formaldehyde are effective with hair and wool Introduction of detergents is potentially hazardous because many of these substances increase the respiratory infectivity of spores (Barnes 1947) Recently developed preparations of antigen are well tolerated and appear to be highly effective in man it is probable that the incidence of industrial anthrax can be greatly reduced by immunization of occupationally exposed workers (Brachman *et al* 1962)

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able results provide no basis for estimating the possible effectiveness of earlier therapy Experimental respiratory infection in monkeys may be suppressed by daily administration of penicillin, but *the animals die of* anthrax if the penicillin is discontinued after 10 days If active immunization is carried out while the infection is suppressed by penicillin the animals survive (Henderson *et al* 1956)

EPIDEMIOLOGY

Although anthrax is primarily a disease of cattle sheep, horses and goats virtually all animals show some susceptibility and the disease has been detected in nearly every country It is particularly common in the Mediterranean region in Africa and in Asia Epizootics with heavy loss of livestock occur at intervals in enzootic areas In Iran during *1945 for example, 1 million of 15 mil*lion sheep were reported to have died of anthrax (Delpy and Kaweh 1946)

Occasional outbreaks are recorded in most sections of the United States but the disease is not a continuing problem except in certain areas During the period 1945 to 1954 3 447 outbreaks were reported from 39 states with loss of 17 600 head of livestock (Stein and Van Ness 1955) The 3 important endemic areas are the Gulf Coast region of Louisiana and Texas a portion of eastern South Dakota and Nebraska and an area in central California The disease was introduced during early settlement and cases in animals and man were recorded as early as 1824 (Hanson 1959) The nature *of the soil and the climatic conditions evi*dently determine the establishment of the disease in certain areas and the periodic occurrence of epizootics (Minett 1952 Van Ness and Stein 1956)

In animals the disease is transmitted primarily by ingestion of contaminated forage or the carcasses of infected animals The bacilli in the unopened carcass are killed rapidly by putrefactive *processes but sporu*lation may occur rapidly if the bacilli are exposed to air Spores may be disseminated over wide areas by surface water animals *birds and insects* Contaminated foods are *frequently* responsible for introduction of anthrax into new areas imported bone meal was the cause of the extensive outbreak in the midwestern United States in 1952 (Stein *and Stoner, 1953) It is not clear what im*portance should be ascribed to saprophytic growth of the organism in favoring persistence of the disease in certain areas The spores may remain viable for many years in soil and some authorities consider that the epidemiology of the disease can be accounted for without postulating saprophytic growth of the organism However *B anthracis* grows readily on ordinary laboratory media and its growth requirements are no more complex than those of typical saprophytic members of the genus (Proom and Knight 1955) There would appear to be no reason why the organism should not grow saprophytically under favorable conditions although antibiotics elaborated by other *microbes may limit its proliferation in soil* (McCloy 1951)

The average number of cases of human anthrax reported annually in the United States during the period 1951 to 1960 was 33 additional unreported cases also occur However the incidence is much greater in many countries and it has been estimated that from 20 000 to 100 000 human cases occur annually in the world (Glassman 1958) Man is infected by contact with infected animals (agricultural anthrax) or contaminated animal products (industrial anthrax) Agricultural anthrax occurs in farmers veterinarians and slaughterhouse workers Industrial anthrax occurs primarily in persons whose work brings them *in contact with* contaminated hair wool or hides although other products have been responsible for scattered outbreaks Occasionally dock workers are infected while handling contaminated cargoes Industrial anthrax in the United States occurs predominantly among workers in textile mills that process goat hair imported from the Middle East (Brachman and Fekety 1958) *Outbreaks of intestinal anthrax are reported* from primitive areas in which meat from animals that have died of the disease is used as food and it is probable that such outbreaks are not uncommon (Nhonoli 1960)

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23

The Clostridia

Organisms of the genus *Clostridium* are anaerobic or micro aerophilic gram positive rods producing endospores which in many species are wider than the rods from which they arise A number of species decompose proteins or ferment carbohydrates Several are pathogenic and a number produce toxins Clostridia are always present in soils and in the intestinal tracts of animals and man The majority are saprophytic and actively decompose organic matter in the soil Some of these (*C acetobutylicum*) are used in industry for the production of chemicals such as acetone and butanol

The pathogenic clostridia include (1) the virtually noninvasive saprophyte *C botulinum* which is dangerous because of the potent toxin it produces in foods (2) *C tetani* which has slight invasive powers but produces so potent a toxin that even slight lodgement in the host is dangerous and (3) frankly invasive clostridia which cause gas gangrene and enterotoxemias in man and animals

MORPHOLOGY

All species consist of relatively large gram positive rods Some (e g *C tetani*) are easily decolorized and retain the stain only when young The pathogenic clostridia vary in size from the large rods of *C novyi* Type B (1 0 — 1 8 × 8 — 12 microns) to the more slender *C tetani* (0 3 — 0 8 × 4 — 8 microns) Cultures may show a great diversity of shapes and pleomorphic forms including filaments orgonts and citrons are characteristic of species such as *C septicum* and *C chauvoei* All clostridia form spores which are usually of greater diameter than the rods from which they arise The shapes and the positions of the spores vary considerably from species to species and these differences are of great assistance in identification and classification Prevot (1948) goes so far as to define orders on the basis of spore position Some species form spores freely in the media usually available while others rarely sporulate This is due to variations in reaction to the nutritional environment rather than to inherent differences in ability to sporulate because media can be devised in which even *C perfringens* will produce dense crops of spores Most clostridia possess peritrichous flagellae and are actively motile However *C perfringens* is consistently nonflagellated and nonmotile A few species (e g *C perfringens*) form capsules

Bacteriologists familiar with aerobic organisms will be struck by the confusing pleomorphism of the clostridia Not only may a variety of forms appear in a particular culture but also their frequency may vary from culture to culture from medium to medium and from strain to strain of the same species However variations in form in a culture should not be dismissed too lightly as manifestations of pleomorphism It has often happened that cultures submitted for typing or identification because of some unusual features could be resolved into mixtures of two or even more species or types

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CULTIVATION

Clostridia are by definition anaerobic or micro aerophilic The extent to which free oxygen inhibits growth varies with the species *C tetani* will not form surface colonies if the air pressure exceeds 4 5 mm of Hg while *C perfringens* will tolerate up to 90 mm (McLeod 1930) *C histolyticum C tertium* and *C carnis* are able to produce visible colonies on *aerobic* blood agar plates

The reasons why clostridia cannot tolerate appreciable amounts of free oxygen are not clear It is known that their metabolic activities require a low oxidation reduction potential (Eh) and that they can achieve this only if the concentration of free oxygen is low It has also been suggested that the deficiency of peroxidases and catalase in clostridia could lead to the formation of lethal quantities of peroxides in the presence of free oxygen (McLeod and Gordon 1925 Gordon Holman and McLeod 1953 Holman 1955) Indeed it might be significant that *C novyi* which forms considerable amounts of peroxide on exposure to air dies rapidly under aerobic conditions According to this very plausible hypothesis it should be possible to protect anaerobes from the effects of peroxides with pyruvate or with enzymes such as peroxidase or catalase However such additions have not in general afforded the expected protection The catalase used successfully by Holman (1955) was a crude extract of fresh ox liver so that its growth promoting effect could well have been attributed to other substances It is remarkable that the gross difference in the reactions of aerobic and anaerobic organisms to oxygen should still remain virtually unexplained

When the Eh of a liquid medium has been reduced to the level where growth can start the growing organism will keep its milieu reduced and multiplication will occur readily provided that the medium is adequate in other respects The reducing conditions necessary are easily obtained in liquid media if the exposed surface is small *in relation to* the volume and if the medium is deaerated immediately before inoculation by boiling for about 10 minutes More exacting anaerobes may require the addition of a reducing sugar (glucose 0 2 to 1%) or reduced iron More efficient reductants such as cysteine (0 01 to 0 1%) sodium thioglycollate (0 01 to 0 1%) or sodium formaldehyde sulfoxylate (0 01 to 0 05%) may be used to provide suitably reduced conditions for the most fastidious clostridia However it must be remembered that the higher concentrations of the thiol containing reductants especially if heat sterilized are inhibitory to some strains Any of the pathogenic clostridia will grow in nutritionally adequate media to which chopped meat or brain have been added The reducing systems involved are discussed by Lepper and Martin (1929) and Hewitt (1962)

For studying colonial morphology or for purifying mixed cultures growth on or in solid medium is necessary Separate colonies of any clostridium can be obtained in deep agar shake cultures or in Veillon tubes However such cultures may be difficult to purify and for this and other reasons growth on surface plates is usually preferable even though conditions suitable for fastidious strains are more difficult to obtain A number of methods have been described for obtaining anaerobiosis for surface growth The simplest most efficient and most widely used is incubation in a MacIntosh and Fildes anaerobic jar or its modification the Brewer jar In these the air is displaced by hydrogen and any residual oxygen is removed by combination with the hydrogen This reaction is catalyzed by palladinized or platinized asbestos contained in an electrically heated gauze cage within the jar Palladinized alumina catalysts (Deoxo) have now become available which are sufficiently active in the cold to obviate the need for a heating coil Jars* fitted with cold catalysts are simpler to use and safer than the conventional jars The growth of many clostridia is improved by the addition of about 5 per cent CO to the hydrogen

All the clostridia described as being pathogenic to man will grow on the surface of blood agar plates incubated in an anaerobic jar (see Fig 1) Some clostridia such as *C novyi* Type B and *C hemolyticum* (which are mainly of veterinary interest) are more exacting than those usually considered pathogenic for man and may require special media to ensure surface growth It may help to pour a thin layer of agar over the surface of an inoculated plate or to add peptones contain

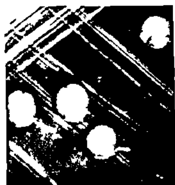
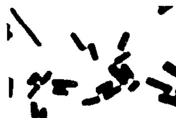
Obtainable from Baird and Tatlock London

TABLE 1 MORPHOLOGY COLONIAL APPEARANCES AND SOME TYPICAL REACTIONS OF PATHOGENIC AND RELATED CLOSTRIDIA*

Organisms	Colonial Appearances							Diameter			Morphology: Vegetative Cells							Morphology: Spores						Precipitate on Egg Agar				
	Smooth	Intermediate	Rough	Fine Rhizoidal	Coarse Rhizoidal	Swarming	Sometimes Motile	3-6 mm	1-3 mm	< 1 mm	Long Slender	Long Thick	Short Slender	Short Thick	Pleomorphism	Capsulation	Motility	Frequency	Swelling Rod	Terminal	Subterminal	Spherical	Oval	Cylindrical	Opalescence	Iridescent Layer	Microaerophilic	Pathogenicity Man	Pathogenicity Animals
Toxigenic Group																													
C. botulinum A B F		X	X			(X)		X	X		X		X				X	++	X		X		X		++	X	0	X	(X)
C. botulinum D C E			X	X	X		X	X	X			X					X	+			X			X	++	X	0	X	X
C. tetani			X	X		X					X						X	++		X		X			0	0	0	X	X
Gas gangrene Group																													
C. chauvoei	X	X				(X)			X	X	X	X			+++		X	++	X		X		X		0	0	0	0	X
C. histolyticum		X							X	X			X				X	++	X		X		X		0	0	X	X	?
C. novyi A		X	X				X	X				X			+		X	++	(X)		X		X		++	X	0	X	0
C. novyi B			X	X	X	(X)	X	X				X			+		X	++	(X)		X		X		+++	0	0	X	X
C. novyi C			X	X	X			X				X			+		X	++	(X)		X		X		0	0	0	0	X
C. novyi D		X							X			X					X	+	(X)		X		X		+++	0	0	0	X
C. perfringens A	X							X						X		X	0	(+)			X		X		+++	0	0	X	?
C. perfringens B	X							X						X		X	0	(+)			X		X		+++	0	0	0	X
C. perfringens C	X							X						X		X	0	(+)			X		X		+++	0	0	X	X
C. perfringens D	X							X						X		X	0	(+)			X		X		+++	0	0	?	X
C. perfringens E	X							X						X		X	0	(+)			X		X		+++	0	0	?	X
C. septicum			X		X	X		X			X	X			+++		X	++	X		X		X		0	0	0	X	X
C. sordellii		X	X			X		X						X			X	+++			X			X	++	0	0	X	X
Associated Species																													
C. bifermentans	X	X				(X)		X						X			X	+++			X			X	++	0	0	0	0
C. butyricum	X							X			X						X	++	X		X		X		0	0	0	0	0
C. fallax		X							X				X			X	X	(+)	X		X		X		0	0	0	0	(X)
C. sporogenes			X		X			X					X				X	+++	X		X		X		++	X	0	0	0
C. tertium	X	X							X		X						X	++	X	X			X		0	0	X	0	0
Rare Species																													
C. capitovale	X									X			X				X	++	X	X			X		0	0	0	0	0
C. cadaveris	X									X			X				X	+	(X)		X			X	0	0	X	0	X
C. cochlearium	X								X				X				X	++	X	X			X		0	0	0	0	0
C. difficile		X						X				X					X	++			X			X	0	0	0	0	(X)
C. sphenoides	X									X			X†				X	++	X	X		X			0	0	0	0	0
C. tetanomorphum	X	X						X			X						X	++	X	X		X			0	0	0	0	0

X The organism possesses the particular property indicated (X) The property in question is possessed by very few strains or possessed to a slight degree 0 The particular property indicated is invariably absent. +++ ++ + The degree to which a particular property is present (+) Rarely present or present to a slight extent only
The descriptions of the different characteristics must be regarded as approximate since it is impossible in such a table to indicate the considerable variation from the mode which will be encountered

† Cuneiform when sporulating.

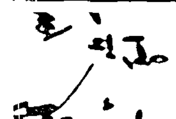
C perfringens blood agar 48 hrs



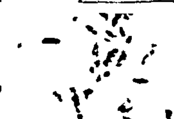

C histolyticum blood agar 48 hrs



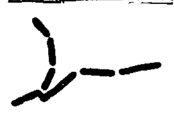

C tertium blood agar 24 hrs

C septicum blood agar 24 hrs

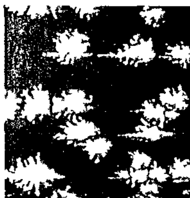
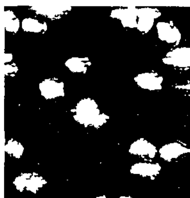
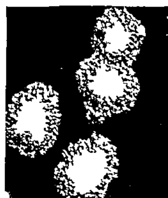
C chauvoei blood agar 48 hrs

C novyi Type A stiff blood agar 48 hrs

FIG 1 (*Continued*)

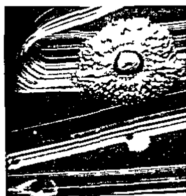
ing high molecular weight polypeptides Fresh batches of medium should be tested with an exacting species such as *C hemolyticum* to make certain that they will support the growth of any pathogenic clostridium which might be encountered

C sordellii stiff blood agar 24 hrs

C histolyticum blood agar 48 agar 24 hrs

C sporogenes blood agar 48 hrs








C botulinum Type B (ovolytic) stiff blood agar 48 hrs

C botulinum Type E (nonovolytic) stiff blood agar 48 hrs

C tetani stiff blood agar 48 hrs

FIG 1 All colonies × 6 all organisms × 2 500

ISOLATION AND IDENTIFICATION

As the constitution of media available in different laboratories varies widely the list given here should be considered as indicating broadly the type of reagents needed Most are procurable commercially or can be made up from commercially available ingredients

Chopped Meat Medium This consists of infusion broth to which have been added meat particles prepared by extracting lean minced heart with water The infusion broth is filled into tubes to a depth of about 2 inches and about one third by volume of the meat particles is added The pH is adjusted so that it will be about 7 4 after autoclaving Glucose reducing agents growth factors etc may be added if required

Fermentation Reactions To 2 per cent proteose peptone and 0 5 per cent NaCl add agar and sodium thioglycollate to 0 1 per cent, adjust pH and autoclave Before inoculation add sugars sterilized by filtration As heat sterilized sodium thioglycollate may be inhibitory it is often advisable to add this before inoculation as a filtered solution The indicator (bromthymol blue) is added when the tests are read

Production of Indol The fermentation medium plus 0 2 per cent sodium phosphate and 0 1 per cent dextrose can be used It can also be used for the vanillin violet reaction particularly if extra tryptophan is supplied

Reduction of Nitrate The fermentation medium with 0 1 per cent potassium nitrate

Liquefaction of Gelatin The fermentation medium plus 5 per cent gelatin

Protein Digestion Add 5 to 8 per cent fresh egg white or 10 per cent serum to infusion broth Mix in a blender dispense in tubes after the foam has subsided Autoclave

Milk Add 5 per cent skim milk to infusion broth adjust pH to 6 8 dispense in deep tubes with reduced iron or iron strips Autoclave

Blood Agar Plates Add 5 per cent fresh defibrinated blood to a suitable agar base The species of blood added has a significant effect on certain hemolytic reactions Double strength agar may be used for preparing stiff antiswarming plates

Egg Plates for Lecithinase Action Collect the yolk of an egg aseptically and mix with an equal volume of physiologic saline Add to a suitable agar base in the proportion of 1 to 10 Kaufman and Weaver (1960a) described methods for the rapid performance of biochemical and fermentation tests and also stated (1960b) that colonies of clostridia fluoresced when grown on media incorporating neutral red so that they could be distinguished from colonies of facultative and of nonsporing anaerobes even on heavily contaminated plates Ingenious combinations of reagents have been used by Willis and Hobbs (1958 1959) to prepare discriminating and selective media For example lecithinase lipolytic proteolytic and saccharolytic properties could be determined on the same plate The often recommended addition of antibiotics to suppress nonclostridial concomitants has its attractions but some inhibition of the development of clostridial colonies especially of field strains may occur

Specific Antitoxic Sera It is virtually impossible to characterize the pathogenic clostridia completely without using antisera The minimum requirements are antisera against *C perfringens* Type A *C novyi* Types A or B *C septicum* *C tetani* and *C botulinum* (all types) If possible antisera against *C perfringens* β, ϵ and ι toxins *C novyi* β and γ lecithinases and *C histolyticum* should also be obtained

Specific anticlostridial sera coupled with fluorescent dyes have recently become available and are invaluable aids to the recognition and the differentiation of clostridial species and types

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The decision as to whether or not a particular clostridium caused a particular condition is ultimately a clinical one However the bacteriologist should attempt to assess the number and the significance of the organisms present in order to provide the clinician with information that will help his decision A bald report that a number of specified clostridia were present is of limited value For this reason there are disadvantages in transporting clinical material in inoculated bottles of holding medium (Wetzler *et al* 1956) since the relative numbers of organisms found in these cultures may differ significantly from those of the original material

Material submitted for examination will

Table 2 Biochemical Reactions of Pathogenic and Related Species of Clostridium

Species	Milk	Dextrose	Maltose	Lactose	Salicin	Sucrose	Gelatin Liquefaction	Nitrate Reduction	Indol	Vanillin Violet	
C butyricum	ACGS	+	+	+	+	+	−	−	−	−	
C perfringens	ACGS	+	+	+	∓	+	+	+	−	−	
C botulinum (non ovolytic Types B C D E)	A	+	+	−	+	∓	+	−	−	−	Carbohydrates variable
C carnis	AG	+	+	+	+	+	−	−	−	−	Micro aerophilic
C capitovale	AC	+	−	−	−	−	+	−	−	−	
C fallax	ACG	+	+	∓	∓	∓	−	−	−	−	
C chauvoei	ACG	+	+	+	−	+	+	+	−	−	
C paraputrificum	ACG	+	+	+	+	+	−	±	−	−	
C septicum	ACG	+	+	+	+	−	+	+	−	−	
C sphenoides	ACG	+	+	+	+	±	−	±	∓	−	
C tertium	ACG	+	+	+	+	+	−	+	−	−	Micro aerophilic
C botulinum (ovolytic Types A B F)	AD	+	+	−	±	−	+	−	−	+	Carbohydrates variable
C bifermentans	CD	+	+	−	+	−	+	−	+	−	Urease − sorbitol +
C histolyticum	CD	−	−	−	−	−	+	−	−	−	Micro aerophilic
C novyi Type B	D	+	+	−	−	−	+	−	−	−	Fastidious
C sordellii	CD	+	+	−	−	−	+	−	+	−	Urease + sorbitol −
C sporogenes	D	+	+	−	−	−	+	+	−	+	Nitrates rapidly reduced but no nitrites produced
C novyi Type A	CG	+	+	−	−	−	+	−	−	−	
C tetani	C	−	−	−	−	−	+	+	+	−	Nitrates rapidly reduced Nitrites absent
C cochlearium	−	−	−	−	−	−	−	−	−	−	
C difficile	−	+	−	−	+	−	−	−	−	−	
C tetanomorphum	−	+	+	−	−	−	∓	−	−	−	

Note A acid C clot D digestion G gas S stormy fermentation + positive − negative ± usually positive ∓ usually negative
No attempt has been made to indicate the intensity of the reactions

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Material submitted for examination will

TABLE 2 BIOCHEMICAL REACTIONS OF PATHOGENIC AND RELATED SPECIES OF CLOSTRIDIUM

SPECIES	MILK	DEXTROSE	MALTOSE	LACTOSE	SALICIN	SUCROSE	GELATIN LIQUEFACTION	NITRATE REDUCTION	INDOL	VANILLIN VIOLET	
C butyricum	ACGS	+	+	+	+	+	−	−	−	−	
C perfringens	ACGS	+	+	+	∓	+	+	+	−	−	
C botulinum (non ovolytic Types B C D E)	A	+	+	−	+	∓	+	−	−	−	Carbohydrates variable
C carnis	AG	+	+	+	+	+	−	−	−	−	Micro aerophilic
C capitovale	AC	+	−	−	−	−	+	−	−	−	
C fallax	ACG	+	+	∓	∓	∓	−	−	−	−	
C chauvoei	ACG	+	+	+	−	+	+	+	−	−	
C paraputrificum	ACG	+	+	+	+	+	−	±	−	−	
C septicum	ACG	+	+	+	+	−	+	+	−	−	
C sphenoides	ACG	+	+	+	+	±	−	±	∓	−	
C tertium	ACG	+	+	+	+	+	−	+	−	−	Micro aerophilic
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C bifermentans	CD	+	+	−	+	−	+	−	+	−	Urease − sorbitol +
C histolyticum	CD	−	−	−	−	−	+	−	−	−	Micro aerophilic
C novyi Type B	D	+	+	−	−	−	+	−	−	−	Fastidious
C sordellii	CD	+	+	−	−	−	+	−	+	−	Urease + sorbitol −
C sporogenes	D	+	+	−	−	−	+	+	−	+	Nitrates rapidly reduced but no nitrites produced
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C tetani	C	−	−	−	−	−	+	+	+	−	Nitrates rapidly reduced Nitrites absent
C cochlearium	−	−	−	−	−	−	−	−	−	−	
C difficile	−	+	−	−	+	−	−	−	−	−	
C tetanomorphum	−	+	+	−	−	−	∓	−	−	−	

NOTE A acid C clot D digestion G gas S stormy fermentation + positive − negative ± usually positive ∓ usually negative
No attempt has been made to indicate the intensity of the reactions

TABLE 3 CLOSTRIDIAL FLORA OF GAS GANGRENE

	PER CENT OF CASES			
ORGANISM	1943 MacLennan (146 Cases)	1944 MacLennan (17 Cases)	1944 Stock (25 Cases)	1946 Smith and George (110 Cases)
C perfringens	56	83	80	39
C novyi	37	47	48	32
C septicum	19	24	4	
C histolyticum	6	6		
C tetani	13		8	4
C bifermentans	4	35	20	54
C sporogenes	37	50	72	54
C tertium	30	59	8	3
C multifermentans				5
C butyricum	13		4	3
C capitovale	5			3
C fallax	1		4	3
C cochlearium	9		4	2
C putrificum	19			2
C regulare				2
C sphenoides	3			2
C paraputrificum				1
C hastiforme	3			
C tetanomorphum	2			

Smith (1955) and Willis (1960) should be consulted for more comprehensive reviews of diagnostic and other clinical procedures

DISEASES CAUSED BY CLOSTRIDIA

Except during wars clostridia play a relatively minor role as agents of disease in man They are found mainly in wound infections (gas gangrene and tetanus) in enterotoxemia (*C perfringens* Types C and F) and in food poisoning (*C perfringens* Type A and *C botulinum*) Most clostridial infections of man are complications of wounds in which vascular damage has favored the development of anoxic conditions suitable for anaerobic growth Outbreaks of food poisoning occur as a result of exceptionally favorable conditions for certain clostridia These outbreaks are self limiting and the affected persons do not act as foci of further infections This is in sharp contrast with the situation among domestic animals in which diseases caused by clostridia are well-defined clinical entities causing constant and serious losses unless combated by the conventional methods of disease control

A greater awareness of the existence of well-defined ecologic groups of pathogenic clostridia within different species might result in further types being found which were capable of provoking specific clinical syndromes in man—such as the food poisoning caused by heat resistant *C perfringens* Type A and enteritis necroticans caused by *C perfringens* Types C and F The finding of *C perfringens* Type D in man and the detection of antibody to the iota toxin of *C perfringens* Type E in soldiers in Korea may indicate a possible pathogenic role for these organisms also

The epidemiologic and epizootiologic characteristics of pathogenic clostridia are correlated with the type of toxin they produce Because of this more attention has been paid to the study of toxins than to the study of the less obviously important antigens such as agglutinogens A number of workers (Moussa 1959 Meisel and Rymkiewicz 1959 Walker and Batty 1962) have shown that the various species of clostridia can be subdivided into types based on differences in somatic flagellar *capsular and* spore antigens Further work on such antigens would extend our knowledge of intraspecific differences in clostridia and would also throw more light on interspecific group

usually be from patients (e g exudates tissues) or articles such as clothing or dressings or suture material suspected of being sources of tetanus or gas gangrene and foodstuffs suspected of causing botulism or other food poisoning The methods of isolation described below apply mainly to clinical material

1 Gram stained films should be made from different parts of the specimen in order to gain an impression of the distribution and the relative numbers of the organisms present In clostridial myositis, films and cultures should be made from the affected muscle rather than from exudate or edema as some highly toxigenic species may not be very apparent unless the actual infected focus in the muscle has been located and filmed

2 Plate directly onto blood stiff blood (antiswarming) and egg yolk agar plates and incubate anaerobically and aerobically for 48 hours The jar should not be opened at 24 hours since even a short exposure to air may inhibit further development of micro colonies of some organisms (e g *C novyi*) A separate jar should be put up for examining at 24 hours if this is considered essential

3 Inoculate several tubes of chopped meat medium Heat some at 80 C for 10 minutes incubate for 24 hours and plate from these cultures as in (2) These plates will not reflect the relative numbers of the different clostridia present in the original material as the chopped meat medium will select the more aggressive species

Plates from (2) and (3) should be examined in detail with a colony microscope and films of putative clostridial colonies should be stained Table 23 2 summarizes the colonial and the microscopic appearances and the reactions on blood and egg agar of the clostridia most likely to be encountered It should be remembered that colonial morphology on stiff agar plates is usually atypical It may be possible to fish selected colonies directly into chopped meat medium but it is always advisable to restreak the selected colony onto a plate in case the one fished into meat broth is impure Several streakings may be necessary before purity is assured

4 Inoculation of laboratory animals—usually guinea pigs—with clinical material triturated with 1 per cent of $CaCl_2$ will assist considerably in detecting and isolating pathogenic clostridia especially when the postmortem appearances point to a particular infecting organism Specific antisera can be used to block one organism or another in a mixed infection The heart s blood and the local lesion of dying or recently dead experimental animals should be cultured as shown in (1) (2) and (3) It should not be assumed that a particular clostridium is pathogenic because it is isolated in large numbers from the heart s blood For example a guinea pig inoculated with a mixture of *C novyi* and *C bifermentans* will die of a *C novyi* infection although the circulation may contain mainly *C bifermentans*

5 The examination of dressings soils etc is carried out in a way basically similar to that used for clinical material the chief differences being in the methods used for obtaining representative samples of the specimen for culturing and animal inoculation Foodstuffs should be examined for the presence of toxins as soon as possible since delay may result in destruction of toxin and the loss of a chance of demonstrating conclusively the role of the food

6 When pure cultures of clostridia have been isolated a tentative recognition can be attempted from the results of reactions in various media (see Table 2) Final identification depends on the production and the identification of toxins and the demonstration of pathogenicity in laboratory animals It must be emphasized again that the presence of known pathogens in tissue or in food is presumptive, but not conclusive evidence of clinical infection

The more general provision of anticlostridial sera labeled with fluorescent dyes will profoundly modify the force of much that has been written above Such reagents make possible the recognition of clostridia in pathologic material (films sections clothing etc) and can indicate the distribution and the numerical relationships of different organisms For many purposes nothing more will be required Batty and Walker (1963a 1963b) have prepared coupled antisera against *C chauvoei C septicum C novyi C tetani* and *C botulinum* It is difficult to overemphasize the simplification that such reagents can bring into diagnosis and their importance in studies on pathogenesis and epizootiology

reactions Smith (1955) should be consulted for discussions of important earlier work on this subject

GAS GANGRENE

About 30 per cent of war wounds become contaminated with clostridia If the contamination is confined to the surfaces of the injured parts little toxin is formed and the results are usually no more than a slight retarding of the rate of healing Anaerobic cellulitis —a spreading infection of the fascial planes—is rather more serious This results in a varying usually slight degree of toxemia but the infection has little tendency to invade healthy tissue and is as a rule self limiting and easily treated In 5 per cent or less of wounds contaminated with clostridia true gas gangrene develops The organisms colonize injured muscle especially where damage to the blood supply has occurred The damaged anoxic muscle provides favorable conditions for multiplication and toxin production and a rapid invasion and destruction of neighboring healthy muscle follows Since the host reaction in the muscle is negligible this condition is more correctly described as a myonecrosis than a myositis The prognosis is serious and speedy and energetic treatment is essential It should be clearly understood that gas gangrene is not synonymous with clostridial wound infection so that detection of clostridia in a wound is not diagnostic of gas gangrene and not necessarily prognostic of a serious outcome Table 3 (from Smith 1949) summarizes the findings of several investigators on the clostridial flora of gas gangrene

C perfringens C novyi and *C septicum* are recognized as the organisms mainly concerned in provoking gas gangrene There are almost certainly in these species intraspecific differences that influence or determine invasiveness and other qualities pertinent to pathogenicity Characteristic toxins and enzymes are produced which play roles of varying significance in invasiveness pathogenicity and virulence

The relation of other clostridia to gas gangrene is somewhat doubtful The toxigenic *C histolyticum* has been found very occasionally in gas gangrene usually in association with other clostridia *C sordellii* (also a toxin producer) has been found as the sole cause of fatal postoperative infections *C sporogenes* and *C bifermentans* are found in the majority of anaerobic wound infections Neither is pathogenic for laboratory animals in pure culture except insofar as occasional strains of *C bifermentans* will markedly digest tissues around the inoculation site and cause lesions resembling those produced by *C histolyticum* but not accompanied by toxemia or followed by death Other clostridia found occasionally in wounds are not considered to be of much significance with the exception of *C tetani* which will be discussed separately See Reed and Orr (1943) Smith and George (1946) Smith (1949 1955) Oakley (1954) and Maclennan (1962)

Clostridium Perfringens

This is the species most frequently associated with anaerobic cellulitis and gas gangrene in man. It is divided into Types A B C D and E according to the main lethal toxin produced while Type F (the cause of enteritis necroticans in man) was separated on account of its heat resistance morphologic peculiarities and differences in minor antigens The main lethal toxins of the various *C perfringens* Types are shown in Table 4 A tabulation of the major and the minor toxins (based largely on Oakley and Warrack 1953) and of the ecologic relationships of types and subtypes of *C perfringens* is given by Brooks *et al* (1957) and Sterne and Warrack (1964) Up to the present, only Type A has been found in anaerobic wound infection of man This type is probably not homogeneous and there is little doubt that it will be possible one day to distinguish subgroups or varieties of epidemiologic significance

Toxins and Enzymes

α toxin A culture of *C perfringens* Type A grown under optimal conditions for toxin production may contain up to 600 mouse LD_{50} per ml (Adams and Hendee 1945) This toxicity is due mainly to the lethal hemolytic and necrotizing α toxin although several other toxins and enzymes notably θ

Table 4 The Distribution of Antigens and Heat Resistance Among the Different Types of Clostridium Perfringens

Type	Occurrence and Country Where First Described	Major Antigens* α Leth l Necrotizing Cadependent L c th nase	β Lethal Necro-tizing	ε Lethal Necro-tizing	ι Lethal Necro-tizing	Minor Antigens† γ Lethal	δ Lethal Hemolytic	η Lethal	θ Hemolyt c Oxygen labile	κ Col lagenase	λ Pro teinase	μ Hyalu ronidase	ν Desoxy ribo-nuclease	Heat resistance
A	Gas gangrene in man and animals													
	Intestinal commensal in man and animals	+++	0	0	0	0	0	+	++	++	0	++	++	0
	Putrefactive processes in soil etc (United States)													
	Food poisoning (Britain)	+++	0	0	0	–	0	–	+	++	0	+	+++	+++
B	Lamb dysentery													
	Enterotoxemia in foals (Britain)	+++	+++	+++	0	++	0‡	–	++	0	+++	+++	++	0
	Enterotoxemia in sheep and goats (Iran)	+++	+++	+++	0	–	–	–	+++	+++	0	0	+	0
C	Enterotoxemia (Struck) of sheep (Britain)	+++	+++‡	0	0	++	+++	–	+++	+++	0	0	++	0
	Enterotoxemia in calves and lambs (United States)	+++	+++	0	0	–	0	–	+++	+++	0	0	++	0
	Enterotoxemia in piglets (Britain)	+++	+++	0	0	–	0	–	++	++	0	+	+++	0
	Necrotic enteritis in man (formerly Type F)§ (Germany)	+++	+++	0	0	+++	0	–	0	0	0	0	+++	+++
	Necrotic enteritis in man (Papua —New Guinea)	+++	+++	0	0	–	0	–	++	++	0	++	–	0
D	Enterotoxemia in sheep lambs goats bovines and possibly man (Australia)	+++	0	+++	0	–	0	–	+++	++	++	++	++	0
E	Sheep and cattle pathogenicity doubtful (Britain)	+++	0	0	+++	–	0	–	+++	+++	+++	+	++	0

+++ Produced by most strains ++ Produced by some strains + Produced by few strains 0 Not produced by any — Not tested
The major antigens are those defining the type and predominantly responsible for pathogenicity
† The minor antigens are of a lower order of toxicity and of l ttle or no importance in pathogenicity
‡ Occasionally the presence of this antigen must be assumed from the production of the appropriate antitoxin by hyper mmunized horses
§ Type F has been abandoned and the stra ns comprising it transferred here
|| From J Path Bact 88 281 1964

TABLE 5 TOXINS AND ENZYMES OF THE DIFFERENT TYPES OF *Clostridium novyi*

		TOXINS AND ENZYMES					
TYPE	DISTRIBUTION	Lethal necrotizing α	Lecithinase hemolytic necrotizing Lethal β	Lecithinase hemolytic necrotizing γ	O labile hemolysin δ	Irides cence ϵ	Hemolysin ζ
A	Human gas gangrene	+++	−	+	+	+	?−
B	Black disease (necrotic hepatitis of sheep)	+++	+	−	−	−	+
C	Osteomyelitis of buffalo	−	−	?+	−	−	−
D	Hemoglobinurea of cattle	−	+++	−			

enzyme is much less toxic than κ toxin In addition to the lecithinase the collagenase and the hyaluronidase *C perfringens* Type A culture filtrates contain a fibrinolysin (which may be a protease) a desoxyribo nuclease (ν toxin) a neuraminidase (which destroys the influenza virus receptor on red blood cells) and a small amount of an enzyme which inactivates the blood group A substance

CLOSTRIDIUM NOVYI

C novyi is divided into 4 types Table 5 modified from Oakley *et al* (1947) and Oakley (1955) summarizes our present knowledge of these Types A and B are dis tinguished by differences in the lecithinases which they produce (see Table 5) although the factor probably responsible for toxemia and death (α toxin) is the same in both Type A is commonly isolated from human gas gangrene while B is usually regarded as an animal pathogen However the latter is more difficult to cultivate than A and could be overlooked more easily Smith and Claus (1957) found that all proteolytic strains of *C novyi* which they examined were Type B so that this type may have been isolated from man more frequently than is generally thought In animals *C novyi* causes well defined diseases such as necrotic hepatitis of sheep (Type B) osteomyelitis of buffalo (Type C) and hemoglobinurea of cattle (Type D) A heat-stable agglutinogen is common to the vegetative cells of all four types (Batty and Walker 1963b)

Toxins and Enzymes Cultures of *C novyi* Types A or B grown under optimal condi tions for toxin production may contain 100 000 mouse LD_{50} per ml This toxicity is almost entirely due to the lethal and necro tizing α toxin about which little further is known The other toxins produced by Types A and B make only a small contribution to the total toxicity They are the γ and β lecithinases (which distinguish Types A and B immunologically) the δ and the ζ hemo lysins produced only by Type A and ϵ toxin which is responsible for the iridescence shown by Type A strains on egg yolk agar (see Table 1) In the case of Type D (*C hemolyticum*) the β lecithinase is produced in very large amounts and becomes of over riding importance as the characteristic lethal toxin of this type The β and γ lecithinases are similar to the lecithinase of *C perfrin gens* differing from it and from each other immunologically and in the species of red cells which they attack Nothing further is known about the δ the ϵ and the ζ toxins Oakley *et al* (1947) should be consulted for a fuller discussion of the toxins of *C novyi*

CLOSTRIDIUM SEPTICUM

C septicum produces a potent α toxin which is lethal, necrotizing and lytic for human and horse red cells According to Bernheimer (1944) a single toxin is re sponsible for all three manifestations Other workers have not found the same close cor respondence between hemolysis and other activities but this could well be due as

κ- and μ toxins, may also be present (see Table 4) The α toxin is the most important (pathologically considered) of the toxins of *C perfringens* Type A and is one of the few bacterial toxins of which the mode of action can be described in chemical terms It is an enzyme (phospholipase or lecithinase C) which catalyzes the hydrolysis of phosphate bonds with the liberation of phosphorylcholine from the phosphatides lecithin cephalin and sphingomyelin (Macfarlane and Knight 1941 de Gier *et al* 1961) It requires calcium for its activity and is therefore inactive in the presence of ions such as phosphate citrate and fluoride which sequester this metal The lecithinase activity of the α toxin is responsible for the opalescence and the turbidity produced when filtrates of *C perfringens* cultures (all types) are incubated together with a solution of egg yolk or when cultures are grown on the surface of egg yolk agar This opalescence is presumably due to the breakdown of the egg yolk lipoproteins lecithovitellin and lecithovitellinin Lecithinases are also produced by other clostridia e g *C novyi C hemolyticum C bifermentans* and by certain bacilli e g *B cereus* All lecithinases except that produced by *C bifermentans* are immunologically distinct from the α toxin of *C perfringens* Since lecithin is widespread throughout the body the opportunities for attack by the toxin are numerous The hemolysis of red cells is doubtless due to the breakdown of lipoproteins but other factors must also be concerned since the red cells of different species are not equally susceptible to hemolysis although the phospholipids isolated from these cells are equally susceptible to hydrolysis (Macfarlane 1950) Other evidence which shows that the ability to attack lecithin is not the only factor determining toxicity is the fact that the lecithinases of *C bifermentans* show considerably less toxicity per unit of enzyme activity than does the immunologically related *C perfringens* lecithinase (Miles and Miles 1950)

The action of the lecithinase in tissues results not only in generalized necrosis but also, specifically in the inactivation of enzyme systems that are dependent on lecithin in one way or another These include the magnesium activated adenosinetriphosphatase of muscle which has a lecithin prosthetic group (Kielley and Meyerhof 1950) and the succinic dehydrogenase system which requires intact lecithin particles possibly for reasons of spatial configuration (Macfarlane and Datta 1954)

The best partially purified preparations contain about 10 000 mouse LD 0 per mg (van Heyningen and Bidwell, 1948 Roth and Pillemer 1953)

θ TOXIN The θ toxin is lethal hemolytic necrotizing and cardiotoxic and is produced by all types of *C perfringens* except Type F and the food poisoning varieties of Type A It is inactivated by mild oxidizing conditions such as standing in air and is reactivated by reducing agents such as cysteine and thioglycollic acid It is inactivated by cholesterol and its production is inhibited in media containing fat so that little or none will be found in cultures grown in chopped meat media Thus θ toxin resembles the oxygen labile cardiotoxic hemolysins of *C tetani Streptococcus pyogenes Diplococcus pneumoniae* and possibly also the δ toxin of *C novyi* Moreover, it is related to these hemolysins immunologically since antibody to any one of them will neutralize any other The relatively high anti θ titers of many normal horse sera are probably the result of early exposure of these animals to one or more producers of O labile hemolysins The toxin has been purified by Roth and Pillemer (1955)

κ-TOXIN This is a proteolytic enzyme which appears specifically to attack collagen (and its breakdown product gelatin) and no other protein Such extreme specificity in proteolytic enzymes is perhaps unique A partially purified preparation of this enzyme made by Bidwell and van Heyningen (1948) contained 500 mouse LD 0 per mg The toxin can be distinguished readily from the other active products of *C perfringens* by immunologic technics (Oakley *et al* 1946)

μ TOXIN This is an enzyme a hyaluronidase which hydrolyzes hyaluronic acid the intercellular cementing ground substance of tissue As a result of its action on the intercellular cement, the toxin spreads rapidly through the skin and substances injected together with it will also be disseminated rapidly Although referred to as μ toxin this

TABLE 5 TOXINS AND ENZYMES OF THE DIFFERENT TYPES OF *Clostridium novyi*

Type	Distribution	Toxins and Enzymes					
		Lethal necrotizing α	Lecithinase hemolytic necrotizing Lethal β	Lecithinase hemolytic necrotizing γ	O labile hemolysin δ	Irides cence ε	Hemolysin ζ
A	Human gas gangrene	+++	−	+	+	+	?−
B	Black disease (necrotic hepatitis of sheep)	+++	+	−	−	−	+
C	Osteomyelitis of buffalo	−	−	?+	−	−	−
D	Hemoglobinurea of cattle	−	+++	−			

enzyme is much less toxic than κ toxin In addition to the lecithinase the collagenase and the hyaluronidase *C perfringens* Type A culture filtrates contain a fibrinolysin (which may be a protease) a desoxyribonuclease (ν toxin) a neuraminidase (which destroys the influenza virus receptor on red blood cells) and a small amount of an enzyme which inactivates the blood group A substance

Clostridium Novyi

C novyi is divided into 4 types Table 5 modified from Oakley *et al* (1947) and Oakley (1955) summarizes our present knowledge of these Types A and B are distinguished by differences in the lecithinases which they produce (see Table 5) although the factor probably responsible for toxemia and death (α toxin) is the same in both Type A is commonly isolated from human gas gangrene while B is usually regarded as an animal pathogen However the latter is more difficult to cultivate than A and could be overlooked more easily Smith and Claus (1957) found that all proteolytic strains of *C novyi* which they examined were Type B so that this type may have been isolated from man more frequently than is generally thought In animals *C novyi* causes well defined diseases such as necrotic hepatitis of sheep (Type B) osteomyelitis of buffalo (Type C) and hemoglobinurea of cattle (Type D) A heat-stable agglutinogen is common to the vegetative cells of all four types (Batty and Walker 1963b)

Toxins and Enzymes Cultures of *C novyi* Types A or B grown under optimal conditions for toxin production may contain 100 000 mouse LD_{50} per ml This toxicity is almost entirely due to the lethal and necrotizing α toxin about which little further is known The other toxins produced by Types A and B make only a small contribution to the total toxicity They are the γ and β lecithinases (which distinguish Types A and B immunologically) the δ and the ζ hemolysins produced only by Type A and ε toxin which is responsible for the iridescence shown by Type A strains on egg yolk agar (see Table 1) In the case of Type D (*C hemolyticum*) the β lecithinase is produced in very large amounts and becomes of overriding importance as the characteristic lethal toxin of this type The β and γ lecithinases are similar to the lecithinase of *C perfringens* differing from it and from each other immunologically and in the species of red cells which they attack Nothing further is known about the δ the ε and the ζ toxins Oakley *et al* (1947) should be consulted for a fuller discussion of the toxins of *C novyi*

Clostridium Septicum

C septicum produces a potent α toxin which is lethal necrotizing and lytic for human and horse red cells According to Bernheimer (1944) a single toxin is responsible for all three manifestations Other workers have not found the same close correspondence between hemolysis and other activities but this could well be due as

Bernheimer suggested to the presence of other hemolysins Further work is necessary to resolve existing contradictions This organism also produces a deoxyribonuclease (*β* toxin) described by Warrack *et al* (1951)

Moussa (1959) showed that *C septicum* strains could be divided into two types on serologic differences in the heat stable agglutinogen This has been confirmed by Walker (Batty and Walker 1963a) Strains vary widely in toxin production pathogenicity and virulence and more intensive investigations would almost certainly reveal important epidemiologic subdivisions Guillaumie *et al* (1953) showed that horses hyperimmunized with *C septicum* filtrates produced antibody to *C histolyticum α* toxin as well as to *C septicum α* toxin and vice versa This ability of the antisera to neutralize both toxins was due not to separate antitoxins but to the capability of individual antitoxin molecules to attach either toxin (Guillaumie *et al* 1954 Sterne and Warrack 1962)

Clostridium Histolyticum Clostridium Sporogenes Clostridium Bifermentans

C histolyticum although wide spread in nature is not often found in wound infections In MacLennan s (1943) series it was found in 10 of 143 cases but only once in pure culture All 10 cases ended fatally Hall (1945) considers that this organism is frequently overlooked in routine examinations and points out that it is more easily isolated on aerobic than on anaerobic media Smooth strains are pathogenic for laboratory animals The *α* toxin (Oakley and Warrack 1950) is lethal and necrotizing and the *β* toxin is a very active collagenase These are both distinct from the hemolysin

Filtrates of *C histolyticum* cultures contain a variety of proteinases It is possible that their remarkable ability to break down tissue could be used for the débridement of eschars in the treatment of burns (Altemeier *et al* 1951 Berman *et al* 1961)

C sordellii has caused fatal postoperative infections in man (Sordelli 1922) and has also occasionally been responsible for post inoculation accidents in cattle and wound infections of sheep (Smith *et al* 1962) Stewart (1938) concluded that *C sordellii* was a pathogenic variety of *C bifermentans* a view which became generally accepted in the English language literature However French workers maintained that they were distinct species (Prevot and Cordier 1941, Tataki and Huet, 1953) A comprehensive study by Brooks M E and Epps (1959) confirmed the French view that *C sordellii* differed from *C bifermentans* in producing urease and showed that this property was correlated with consistent differences in colonial morphology and fermentation reactions The urease positive strains (*C sordellii*) could be toxigenic or nontoxigenic whereas the urease negative strains (*C bifermentans*) were never toxigenic Only the latter conformed in properties and appearance to the type strain of *C bifermentans* of Tissier and Martelly Pathogenic strains of *C sordellii* produce one—and possibly more than one—powerful lethal toxin which provokes the appearance of a remarkably extensive clear thick edema when injected into laboratory animals Natural cases of *C sordellii* infection are similarly characterized and may resemble infection produced by *C novyi*

C sporogenes and *C bifermentans* are often found associated with other clostridia in anaerobic wound infections Their role in the pathogenesis of gas gangrene is uncertain They cause no toxemia and it is doubtful whether they provoke gas gangrene although they may exacerbate infections by other clostridia On occasion *C sporogenes* has been isolated as the only clostridium in a wound infection However it grows so easily that it would be comparatively easy to overlook an admixture with a fastidious organism such as *C novyi*

Pathogenesis

Gas gangrene is a rapidly spreading myonecrosis of healthy muscle following infection by pathogenic clostridia of severely injured muscle particularly when the blood supply to the muscle has been interrupted The anoxia and the consequent anaerobic glycolysis result in a fall of the Eh to levels permitting multiplication and toxin production by the clostridia A further result is an accumulation of lactic acid and a fall in pH which favors the activity of the catheptic enzymes in the muscle Amino acids thus become available for the clostridia and enable them to grow at a higher Eh than would

otherwise be possible (see Oakley 1954 MacLennan 1962)

Once the anaerobes have gained a foot hold in the damaged muscle they are aided by their extracellular toxins and enzymes in colonizing it and attacking undamaged muscle This is seen most clearly in the case of *C perfringens* Type A which has been the most extensively studied The α toxin breaks down cells containing lecithin and interferes with their metabolism It renders capillaries more permeable to fluid and protein so that the resulting extravasation increases pressure within the muscle thus exacerbating the anoxia By breaking down cells it not only exposes them to attack but also provides more nutrient for the organism Since the production of θ toxin is inhibited in the presence of meat it is unlikely that this toxin plays an important part in the pathology of infection The κ toxin (collagenase) breaks down collagen barriers to the spread of the organism and the destruction of reticulin around capillaries may lead to further interference with the blood supply of the muscle The muscle is disintegrated by the destruction of its reticulin and collagen scaffolding and exposed to the action of catheptic enzymes and bacterial proteases The hyaluronidase (μ toxin) assists the spread of the organism by breaking down the interstitial ground substance and thus providing fermentable carbohydrate in the form of breakdown products of hyaluronic acid

However it appears likely that only the α toxin is essential for the initiation (at least) of the pathologic processes induced by *C perfringens* Evans (1943a 1943b 1947) showed that guinea pigs in which gas gangrene had been produced by injecting *C perfringens* could be saved with α antitoxin while θ κ and μ antitoxins were ineffective alone and did not enhance the effect of α antitoxin when administered together with it It is still possible that the minor toxins may contribute to the development of the pathologic process once this has started However the amounts of α and other toxins which different strains produce in vitro are not directly related to their virulence (Keppie and Robertson 1944 Evans 1945)

The ability of organisms to destroy tissues by means of extracellular enzymes does not necessarily connote a high degree of pathogenicity Some strains of the very proteolytic species *C sporogenes* and *C bifermentans* readily digest living tissue but the infections they cause on injection into experimental animals tend to be self limiting and unaccompanied by toxemia In wound infections the presence of these and other relatively non pathogenic organisms alters the gross pathologic appearance of the lesion elicited by the frankly pathogenic clostridia and may increase the severity of the infection

Infections with *C perfringens* have short incubation periods (9 to 48 hours) The muscle is soft and pulpy and there is little edema Fat droplets may be seen on the surface of the fluid exudate surrounding the affected muscle Erythrocytes are lysed and leukocytes are degenerated There is no putrefactive odor Experimental infection of guinea pigs causes a pink soggy appearance of the muscle On cutting into the lesion a pink stained fatty fluid exudes There is a moderate amount of gas and with some strains quite a marked digestion of the tissues The amount of edema varies considerably with the infecting strain There is little smell of putrefaction Infections with *C novyi* have longer incubation periods (about 5 days) and a higher mortality rate than those with *C perfringens* There is a very marked edema and a profuse serous discharge from the wound (MacLennan 1943) There is little gas and little odor unless other proteolytic clostridia are present In the experimentally infected guinea pig the abdominal wall and the subcutaneous tissues are infiltrated with a transparent colorless or pale pink edema which may be nearly a centimeter thick This edema surrounds the leg in which the injection was given The area immediately around the injection site may be hemorrhagic Small gas bubbles may be present and there is no putrefactive smell Organisms can be seen readily near the site of injection but there are relatively few in the extensive edema *C septicum* is not infrequently associated with gas gangrene in man and may be the only organism present The incubation period is 2 to 3 days There is little gas and considerably less serous exudate than with *C novyi* In guinea pigs death usually occurs within 24 hours The affected muscles are dark red sometimes almost

black in color with some gas at the inoculation site and small bubbles of gas in the flank and along the abdominal wall A bloody edema may extend along the abdominal wall but is not nearly so prominent as that due to *C novyi* The liver is often pale and films made from it show large numbers of organisms Impression films made from the liver surface shortly after death show long chains and filaments of clostridia The mucous membrane of the intestine especially the small intestine is often markedly hemorrhagic and with some strains the lumen may be filled with lysed blood The cadaver has a characteristic rancid smell that is almost diagnostic of *C septicum* or *C chauvoei* infection

While it is clear that the α toxin is largely responsible for the pathogenicity of *C perfringens* and that the gas gangrene induced by this organism is characterized by a profound toxemia it is by no means certain that this toxin is directly responsible for the toxemia even though many lethal doses of toxin may be produced in the infected muscle These doubts arise largely from the work of Macfarlane and MacLennan (1945) and MacLennan (1962) They suggested that the toxemia and the anemia of gas gangrene might be due not to α toxin but to some toxic substance liberated by its action (or by some other action of *C perfringens*) on muscle They showed that *intravenous* injection of α toxin into rabbits led to intravascular hemolysis, but that there was no intravascular hemolysis and no toxin detectable in the circulation after a lethal *intramuscular* injection Furthermore they were unable to recover α toxin from the muscle of injected animals or from gangrenous human muscle They suggested therefore that the toxin remained adsorbed to muscle and that no significant amount entered the circulation to cause systemic poisoning

It could be argued that if the toxin were released slowly into the circulation it would be undetectable and would be too dilute to cause intravascular hemolysis but would yet be sufficient to allow for a lethal accumulation at the susceptible sites in the body However this concept is unlikely in view of the observation that patients could suffer from acute toxemia in spite of high concentrations of antitoxin in the blood That this might have been the result of an irreversible attachment of toxin to susceptible sites before antitoxin administration was improbable because surgical excision of the affected muscle resulted in a dramatic amelioration of the toxemia Therefore, it would seem more likely that a toxemia producing substance passed from the affected muscle to the circulation but that this substance was not α toxin—since it was not neutralizable by specific antitoxin or detectable by any test for α toxin—but a substance produced by the action of α toxin No such substance has yet been isolated

Prophylaxis and Treatment

Potent antitoxins can be prepared by hyperimmunization of horses with toxic filtrates of *C perfringens* *C novyi* *C septicum* and *C histolyticum* In experimental animals a high degree of protection can be achieved by a preceding inoculation with the specific antitoxin In man it is customary to give 10 000 units of *C perfringens* 10 000 of *C novyi* and 5 000 of *C septicum* α antitoxins prophylactically and about 3 times these amounts every 4 to 6 hours for therapy Treatment with antisera appears to be of benefit provided that and only provided that adequate surgery has been carried out (see Macfarlane 1943 MacLennan and Macfarlane 1944) Necrotic muscle must be removed as early and as radically as possible If all damaged muscle can be excised the source of toxemia provoking substances and the bulk of the infecting organisms will be removed with it and the Eh of adequately vascularized muscle will be too high for the remaining clostridia to multiply in it Moreover early removal of devascularized muscle will prevent the fall in pH and consequent proteolysis which results in conditions suitable for clostridial multiplication (Oakley 1954)

However, Brummelkamp *et al* (1963) concluded that surgical intervention could be virtually dispensed with if the patient was allowed to breathe oxygen while under 3 atmospheres of air pressure in a compression chamber The consequent high pressure of oxygen in the tissues was considered to inhibit the spread of clostridia to healthy

muscle It should be noted that the only pathogenic anaerobe isolated by Brummelkamp *et al* in their series of 26 cases was *C perfringens* and this was indeed the only clostridium isolated in 24 of the cases Therefore the effects of high pressure oxygen on gas gangrene caused by other clostridia should be investigated before surgery is—perhaps prematurely—abandoned

There is little evidence of the usefulness of chemotherapy of gas gangrene of man At best it may supplement but not displace surgical treatment The successful active immunization of humans against diphtheria and tetanus (see below) by means of appropriate toxoids naturally encouraged hopes of similar immunization of persons exposed to risk of gas gangrene Work on these lines was initiated by British and American workers during the World War II and it was found that an appreciable antibody response could be elicited in man and animals by the use of aluminum adsorbed formol toxoids (Altemeier *et al* 1952 Robertson and Keppie 1943 Barr *et al* 1945) There are no data on the effects of such active immunization on the subsequent exposure of man to gas gangrene However immunization of domestic animals with *C perfringens C septicum* and *C novyi* vaccines is of demonstrable value against diseases caused by these organisms

TETANUS

Tetanus is a toxemia due to infection of an injury (which may be quite insignificant) with *C tetani* It may also be associated with burns puerperal infections infections of the umbilical stump (tetanus neonatorum) and various surgical procedures in which the infection may originate from contaminated suture materials dressings plaster etc Although tetanus is usually regarded as typically a complication of war injuries the incidence in civil life is not negligible In Great Britain the mortalities in civil life distributed by age show two maxima the first during childhood and the second in later middle life

The incubation period in man is usually between 5 and 10 days which allows for spore germination growth and toxin production and development of evident symptoms of toxemia These are characterized by convulsive tonic contraction of voluntary muscles In man the first symptoms are generally muscular spasms in the region of the local infection followed by trismus which rapidly increases to fixation In some cases the symptoms remain localized near the site of infection

Clostridium Tetani

The tetanus bacillus is widely distributed in soils (20 to 47% of samples) and in animal feces (up to 30%) Therefore wounds contaminated with soil may result in tetanus although this is by no means an invariable consequence of the presence of *C tetani* in a wound Tulloch (1919) for example isolated the organisms from 19 out of 100 wounds in which there was no evidence of tetanus On the other hand it has been reported that spores may be phagocytosed and remain dormant for several months and then initiate a fatal infection if they happen to be deposited in an injured area (contused wounds sites of injection of irritant substances See Smith 1955)

Young actively growing organisms are gram positive but most cultures are completely gram negative by 48 hours and very often by 24 hours The spores are terminal and round when completely ripe but usually appear slightly oval Surface colonies may be surrounded by an area of swarming and this habit can be utilized for the isolation of *C tetani* from mixed cultures (Fildes 1929) Deep colonies are semitransparent and woolly *C tetani* is not as easily grown on the surface as *C perfringens* but is less exacting than *C novyi* It can be divided into 10 agglutinating types on the basis of specific flagellar antigens Batty and Walker (1963b) examined 26 strains from 8 countries and found that all possessed a common heat stable antigen Therefore an antiserum prepared against this could be coupled with a fluorescent dye to provide a specific reagent for the recognition of any strain of tetanus All types produce a neurotoxin neutralized by one antitoxin

Toxin. A culture of *C tetani* grown under optimal conditions for toxin production may contain up to 2 million LD_{50} per ml (Mueller and Miller 1954) but toxin production

black in color, with some gas at the inoculation site and small bubbles of gas in the flank and along the abdominal wall A bloody edema may extend along the abdominal wall but is not nearly so prominent as that due to *C novyi* The liver is often pale and films made from it show large numbers of organisms Impression films made from the liver surface shortly after death show long chains and filaments of clostridia The mucous membrane of the intestine especially the small intestine is often markedly hemorrhagic and with some strains the lumen may be filled with lysed blood The cadaver has a characteristic rancid smell that is almost diagnostic of *C septicum* or *C chauvoei* infection

While it is clear that the α toxin is largely responsible for the pathogenicity of *C perfringens* and that the gas gangrene induced by this organism is characterized by a profound toxemia it is by no means certain that this toxin is directly responsible for the toxemia even though many lethal doses of toxin may be produced in the infected muscle These doubts arise largely from the work of Macfarlane and MacLennan (1945) and MacLennan (1962) They suggested that the toxemia and the anemia of gas gangrene might be due not to α toxin but to some toxic substance liberated by its action (or by some other action of *C perfringens*) on muscle They showed that *intravenous* injection of α toxin into rabbits led to intravascular hemolysis but that there was no intravascular hemolysis and no toxin detectable in the circulation after a lethal *intramuscular* injection Furthermore they were unable to recover α toxin from the muscle of injected animals or from gangrenous human muscle They suggested therefore that the toxin remained adsorbed to muscle and that no significant amount entered the circulation to cause systemic poisoning

It could be argued that if the toxin were released slowly into the circulation it would be undetectable and would be too dilute to cause intravascular hemolysis but would yet be sufficient to allow for a lethal accumulation at the susceptible sites in the body However this concept is unlikely in view of the observation that patients could suffer from acute toxemia in spite of high concentrations of antitoxin in the blood That this might have been the result of an irreversible attachment of toxin to susceptible sites before antitoxin administration was improbable because surgical excision of the affected muscle resulted in a dramatic amelioration of the toxemia Therefore it would seem more likely that a toxemia producing substance passed from the affected muscle to the circulation but that this substance was not α toxin—since it was not neutralizable by specific antitoxin or detectable by any test for α toxin—but a substance produced by the action of α toxin No such substance has yet been isolated

PROPHYLAXIS AND TREATMENT

Potent antitoxins can be prepared by hyperimmunization of horses with toxic filtrates of *C perfringens C novyi C septicum* and *C histolyticum* In experimental animals a high degree of protection can be achieved by a preceding inoculation with the specific antitoxin In man it is customary to give 10 000 units of *C perfringens* 10 000 of *C novyi* and 5 000 of *C septicum* α antitoxins prophylactically and about 3 times these amounts every 4 to 6 hours for therapy Treatment with antisera appears to be of benefit provided that and only provided that adequate surgery has been carried out (see Macfarlane 1943 MacLennan and Macfarlane 1944) Necrotic muscle must be removed as early and as radically as possible If all damaged muscle can be excised the source of toxemia provoking substances and the bulk of the infecting organisms will be removed with it, and the Eh of adequately vascularized muscle will be too high for the remaining clostridia to multiply in it Moreover early removal of devascularized muscle will prevent the fall in pH and consequent proteolysis which results in conditions suitable for clostridial multiplication (Oakley 1954)

However Brummelkamp *et al* (1963) concluded that surgical intervention could be virtually dispensed with if the patient was allowed to breathe oxygen while under 3 atmospheres of air pressure in a compression chamber The consequent high pressure of oxygen in the tissues was considered to inhibit the spread of clostridia to healthy

intramuscularly However, that same intra muscular dose of toxin failed to kill if the regional motor nerves to the injected muscle had been divided previously Thus it is clear that toxin injected into the muscles reaches the central nervous system by a route sheltered from circulating antitoxin in all probability via the motor nerves

A small dose of toxin injected into the hind limb of an animal is absorbed by the motor nerves and travels up the nerve trunk and results in ascending tetanus which affects first the muscles in the injected leg then those of the opposite leg then those of the back and the abdomen When a large dose of toxin is injected into a site from which direct absorption by motor nerves is not possible it first appears in the lymph from the area injected and thence passes into the blood stream from which it is absorbed by all the peripheral motor nerves This leads to descending tetanus where the muscles first affected are those of the head and the neck, followed by those of the arms the trunk and the legs

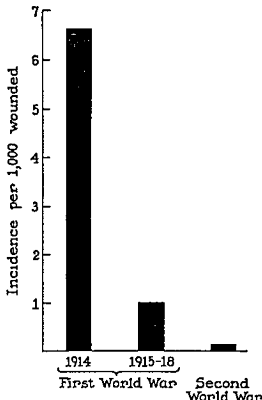


FIG 2 Incidence of tetanus in the British Army in two world wars Prophylactic passive immunization against the toxin was introduced toward the end of 1914 active immunization before World War II (van Heyningen W E 1955 Cambridge University Press)

PROPHYLAXIS AND TREATMENT

Experience with troops during World War I showed clearly the value of prophylactic passive immunization with antitoxic horse serum Experience in World War II showed equally convincingly that tetanus could be eliminated by active immunization of troops with toxoid (see Fig 2) The incidence in World War II was only a tenth of that in the first 35 cases in the British army (15 in nonimmunized soldiers) and only 12 in the American army On the other hand tetanus was relatively frequent and accompanied by a high mortality in the German and the Japanese armies in which active immunization was not practiced

In civilian practice injured persons are usually given prophylactic hyperimmune serum and this confers a high degree of resistance to tetanus for a limited time A complication is that spores of *C tetani* may germinate late in almost healed wounds by which time passive protection may have fallen below the safe limit A further difficulty is that persons who have had previous inoculations of serum may have become immune to it and will eliminate a subsequent injection of antitetanic serum so rapidly that it may prove to be ineffective The success of active immunization of soldiers suggested the desirability of immunizing civilians similarly In 1943 active immunization of young children against both tetanus and diphtheria was made compulsory at Lyons Since then the practice has become almost universal At present a combination of pertussis diphtheria poliomyelitis and tetanus vaccines is widely favored

The results obtained in the treatment of tetanus with antiserum have been poor because it is not possible to dissociate the complex of toxin and the susceptible moiety of nervous tissue Nevertheless antiserum should be given to neutralize toxin which is being formed and has not yet been fixed

varies widely with the strain Although tetanus toxin was the classic example of an exotoxin, it is now known that the young bacilli contain most of the toxin subsequently found in the medium (Raynaud 1947) Tetanus toxin was one of the first to be obtained in a highly purified crystalline state (Pillemer *et al* 1946 1948) It is a simple protein, with a molecular weight of 67,000 containing 70 000 000 mouse LD_{50} per mg Thus tetanus botulinum and dysentery toxins are the most poisonous substances known In the highly purified state the toxin rapidly and spontaneously loses its toxicity but not its antigenicity (i e it toxoids) and at the same time its molecular weight appears to double (Pillemer and Moore 1948) In dilute solution the toxin whether crude or purified is highly susceptible to surface denaturation like many active proteins, and should be protected by the addition of gelatin or broth to the diluent In contradistinction to this inactivation by dilution a number of workers have reported an increase of toxicity (potentiation activation) in solutions of the toxins in diluents containing broth serum organ extracts acetates citrates or proteolytic enzymes (Traub *et al* 1946) Potentiation appears to be demonstrable in some animals but not in others which suggests that the effect may be not on the toxin alone but on its interaction with the host However this work awaits confirmation since the control experiments designed to show that the potentiation was not merely a protection against denaturation do not appear to be conclusive

The toxin is specifically and avidly fixed by the gray matter of nervous tissue (Wassermann Takaki phenomenon) The substance responsible for this fixation is a ganglioside a water soluble lipid containing residues of stearic acid sphingosine glucose galactose N acetylgalactosamine and N acetylneuraminic acid (van Heyningen and Miller 1960) The part that this fixation plays in the lethal action of the toxin has not yet been determined

The main action of the toxin is on the neural elements of the central nervous system where it appears like strychnine to diminish or abolish synaptic inhibition (Brooks V B *et al* 1957) When injected into the anterior chamber of the rabbit s eye it acts on the cholinergic motor nerve of the *sphincter pupillae* of the iris and paralyzes the muscle (Ambache *et al* 1948) Botulinum toxin exerts a similar action (Ambache 1951) possibly by preventing the release of acetylcholine from the motor nerve endings However the actions of these two toxins differ, for tetanus toxin is extremely lethal when injected into the medulla oblongata whereas botulinum toxin has no comparable effect (see Wright 1955)

In addition to the neurotoxin *C tetani* also produces small amounts of an oxygen labile hemolysin which like the other oxygen labile bacterial hemolysins is necrotizing and cardiotoxic

Pathogenesis

C tetani has limited powers of invasion which are not reinforced by the neurotoxin since this has no general histotoxic effect The hemolysin which is necrotizing may possibly play a supporting role Only a slight degree of local tissue destruction is needed for initiating infection and establishing the lowered Eh necessary for germination of the spores (Fildes 1929) Once multiplication has started toxin is formed rapidly and is so potent that quite small amounts will result in a fatal outcome The harmful effects of *C tetani* infections are entirely due to the neurotoxin

The mechanism by which toxin spreads from the site of injection to the susceptible sites in the central nervous system has been the subject of controversy for many years but it now seems certain that toxin travels first up the motor nerve trunks and then up the spinal cord This is essentially the route proposed originally by Meyer and Ransom (1903) Their views were opposed vigorously by Abel (see Wright 1955) who contended that the toxin was carried to the central nervous system exclusively in the blood stream More recent evidence supports the earlier workers For example different workers have shown in different ways (Friedemann *et al* 1941 D Antona 1951) that an intravenous dose of antitoxin which could protect against a given dose of toxin administered intravenously failed completely to protect against a similar dose administered

resemble loose balls of cotton wool. Types A, B and F are far more proteolytic than the virtually nonproteolytic C, D and E. Smith (1955) should be consulted for a more complete discussion of the cultural and biochemical characteristics of this species.

Man is affected mainly by Types A and E, less frequently by Type B, and never (with one possible exception) by C or D. There is a single recorded incident of intoxication with Type F (Møller and Scheibel, 1960). These differences almost certainly reflect variation in human susceptibility to the different types of toxin as much as in degree of exposure to one or another of them. For example, there is a much lower case mortality (20% or less) in Type B than in Type A (70%) intoxications, and recovery from Types B and E has occurred even when appreciable amounts of toxin could be demonstrated in the blood. In some areas (e.g., South Africa) where there is considerable exposure to C and D toxin, no human cases of intoxication have occurred. Only Types C and D have been found in herbivores in nature.

The relatively high resistance of the spores of *C. botulinum* to physical and chemical agencies set a problem for the canning industry where sterilization in bulk is necessary and where excess heating is deleterious to the product. The safety record of commercial canned food is evidence that the problem has been largely solved (Smith, 1955; Tanner and McCrea, 1923). However, an outbreak of botulism caused by commercially canned tuna fish (Johnston *et al.*, 1963) emphasized that slipshod processing remains a hazard. Generally speaking, spores may survive several hours at 100° C. and up to 10 minutes at 120° C. The spores of Type E are much less resistant than those of the other types. As with other clostridia and with bacilli, great variation in resistance is found in different strains and in different cultures of the same strain.

Toxins. Cultures of *C. botulinum* grown in the ordinary way under optimal conditions for toxin production may contain 2,000,000 mouse LD$_{50}$ per ml., and when grown in cellophane sacs suspended in culture medium yields as high as 200,000,000 LD$_{50}$ per ml. have been obtained (Sterne and Wentzel, 1950). Toxin appears to be formed within the organisms and released on autolysis. Production does not seem to depend on active multiplication, since toxin is formed rapidly in resting cells (Kindler *et al.*, 1956).

Each Type of *C. botulinum* is characterized by a toxin immunologically distinct from that of any other type. While hyperimmune sera to Types A and B are monospecific, antisera to Type C usually contain a small amount of D antitoxin and considerably less E antitoxin. Similarly, antisera to Type D may contain a little antitoxin to Type C and less to Type E. Antiserum to Type E may contain some antitoxin to Type D. These cross neutralizations are probably due to possession of moieties of the heterologous toxins by Types C, D and E (Mason and Robinson, 1935; Bowmer, 1962). Although the toxins are immunologically distinct, their pharmacologic effects are identical and are effected through injury to the peripheral nervous system. They act at the myoneural junction, apparently by preventing the release of acetylcholine from the demyelinated ends of the cholinergic motor nerves. There is no effect on the peripheral adrenergic nerves. It is not known how the release of acetylcholine is inhibited, but the evidence suggests that the nerve impulse is stopped in the demyelinated nerve endings somewhere near the point of terminal arborization (Brooks, V. B., 1954). There does not appear to be any destruction of acetylcholine by the toxin, or any interference with the enzymes breaking down the transmitter. (See Wright, 1955, for a comprehensive and stimulating review of the pharmacology of botulinus and tetanus neurotoxins.)

Although the different types of botulinum toxin have the same pharmacologic action, they differ greatly in their relative pathogenicities for different animal species. For example, the ratio of the lethal doses for mice and fowls are 1:15 for Type A, 1:2,000 for Type C, 1:100,000 for Type D and 1:25 for Type E. Moreover, there is considerable variation in the ratio of oral to parenteral toxicity among the different types and for different animals. These differences are almost certainly of major epidemiologic significance.

The botulinum toxins that have been purified all contained about 30,000,000 mouse LD$_{50}$ per mg. Type A was first purified and

Death following injury caused to nerve tissue by tetanus toxin is usually the result of respiratory failure or of starvation due to difficulty in swallowing If these sequelae can be staved off by artificial respiration and feeding and by the administration of relaxant and tranquilizing drugs the patient may recover completely because the injury to nerve tissue is reversible Gratifyingly high cure rates have been achieved when such therapy has been applied by skilled teams (Smith *et al* 1956 Smythe 1963, Anonymous 1963)

BOTULISM

Botulism is not an infectious disease but a poisoning caused by eating food in which *C botulinum* has grown and produced toxin In man symptoms appear after periods varying from less than a day to several days after ingestion of the toxin depending on the amount taken They include vomiting constipation thirst double vision difficulty in swallowing and speaking and respiratory paralysis In survivors convalescence is slow and partial paralysis may persist for several months

A few cases of botulism due to wound infection have been reported (Davis *et al*, 1951 for example) but for all practical purposes the organism may be considered as noninvasive Outbreaks of botulism among humans are sporadic and rare and would be regarded as insignificant were it not for the *high fatality rate From 1899 to 1947 there* were 462 outbreaks in the United States and Canada with 1 253 cases and 815 deaths In the United Kingdom only 4 outbreaks have been recorded In animals botulism occurs far more frequently than in man and may be so prevalent in some areas that husbandry becomes virtually impossible unless measures are taken to control the disease

Human cases result from the eating of smoked salted spiced or canned foods which have been allowed to stand for a period and eaten without being cooked In Europe most cases have been due to sausages (Latin *botulus* whence botulism) ham spiced meats game pastes potted meats and in Russia salt fish In the United States most cases have been due to canned especially home-canned vegetables and fruits such as olives string beans, corn and peas Affected meats may appear obviously spoiled but the growth of *C botulinum in canned vegetables* may be slight and apparently insignificant, although dangerous amounts of toxin may be formed Dack (1949), Tanner and Tanner (1953) should be consulted for further detail

CLOSTRIDIUM BOTULINUM

The natural habitat of the organism is the soil It is rather more prevalent in virgin soil than in cultivated manured or pasture lands In the United States it appears to be more common in the Rocky Mountain system and west of it It occurs less frequently in the states on the Atlantic seaboard and rarely in the Great Plains or the Mississippi River Valley (Meyer and Dubovsky 1922a) Type A (see below) is more common than Type B in the United States while the latter is more usual in Europe (Meyer and Dubovsky 1922b) The frequency with which *C botulinum* occurs in virgin soils and in vegetable matter of various kinds suggests that it is not, as was once thought primarily an *intestinal* parasite It can be found in the feces of domestic animals in areas where botulism is enzootic but it has almost never been isolated from human feces Similarly Type E is the dominant type in for example the Baltic catchment area The frequency with which it can be isolated from the peel of stored potatoes is a strong indication of its tellural origin (Johannsen 1963)

Botulinum organisms are divided into two *species the strongly proteolytic (ovolytic) C parabotulinum* and the less proteolytic (nonovolytic) *C botulinum* They are also divisible into Types A B C D E and F according to the immunologic *specificities of* the toxins they produce There is a considerable diversity of cultural and biochemical behavior within these types so that it is possible to have representatives of both *C botulinum* and *C parabotulinum* in Type B However the immunologic differences in the toxins are constant and clear-cut and since these characteristics are of definitive epidemiologic significance it is now customary to refer to all organisms producing botulinum toxin as *C botulinum* and to subdivide these into Types as recommended at the 6th International Congress of Microbiology (1953) Deep colonies of Types A and B may be round or lenticular while those of C and D

intoxication in which recovery has occurred even after toxin has been demonstrated in the circulation It is rational to think that benefit would result from the neutralization of such circulating toxin Dolman and Iida (1963) strongly recommended treating Type E intoxication with antitoxin As in tetanus the lesion caused by botulinum toxin may regress and the paralyses and other clinical manifestations of the disease gradually disappear For this reason artificial respiration should be maintained for long periods by mechanical means if necessary (Legroux *et al* 1944 Mouquin *et al* 1944)

MISCELLANEOUS INFECTIONS

Clostridium Perfringens Type A

McClung (1945) and Hobbs *et al* (1953) isolated *C perfringens* Type A from cases of food poisoning and from food suspected of causing food poisoning in circumstances which strongly incriminated this organism It has since been found to be associated with a number of similar outbreaks in different parts of the world The characters of the strains isolated are such as to suggest that they form a well-defined ecologic subdivision of Type A It is heat resistant produces little α toxin and no θ toxin

Clostridium Perfringens Type C

Recently Murrell and Roth (1963) in Papua New Guinea described cases some fatal of necrotic enteritis in man caused by *C perfringens* From these a number of strains were isolated by Egerton and typed by Warrack and Walker (Egerton and Walker 1964) and shown to produce α and β toxins In this they resembled Zeissler s isolates However they lacked heat resistance the definitive character of Type F and also differed from this in producing θ hemolysin traces of κ collagenase and hyaluronidase Hence the Murrell and Roth strains must be placed in Type C (Table 23-4) While from the epidemiologic and the pathologic standpoints it would be tempting to group the Papua New Guinea strains with Zeissler s strains in Type F this would be taxonomically inadmissable This difficulty strengthens the already weighty arguments for abandoning Type F and transferring Zeissler s strains to Type C (Sterne and Warrack 1964) The similarities of the antigenic spectra of the Papua–New Guinea strains and the strains isolated by Field and Gibson from piglets in Britain is worth noting (Table 4)

Clostridium Perfringens Type D

This organism which produces ϵ toxin and is the cause of pulpy kidney disease in sheep has been isolated from man twice (Gleeson White and Bullen 1955 Kohn and Warrack 1955) Its significance in man is not known

Clostridium Perfringens Type E

Marshall and Anslow (1955) examined sera from a number of cases of epidemic hemorrhagic fever from Korea and found that several of these had antibody to the ι toxin of *C perfringens* (the main lethal toxin of Type E) It was not suggested that Type E stood in any etiologic relationship to epidemic hemorrhagic fever *C perfringens* Type E was not isolated from any of these cases so that the reason for the ability of these sera to neutralize ι toxin is as yet obscure

Clostridium Perfringens Type F

In 1949 Zeissler and Rassfeld-Sternberg described an outbreak in Northern Germany in which a number of fatalities occurred of an enterotoxemic disease of man characterized by a sloughing necrotic enteritis This disease is analogous to the specific clostridial enterotoxemias of animals and was shown to be caused by a *C perfringens* which produced large amounts of β toxin (Oakley 1949) Because the strain was markedly heat resistant and showed some morphologic peculiarities and because it lacked most of the perfringens minor toxins it was classified as a new type Type F rather than Type C in which its production of β toxin would naturally have placed it. In view of the heterogeneity now known to exist in Type C it might be as well to transfer Type F to this group (Brooks M E *et al* 1957 Sterne and Warrack 1964)

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crystallized by Lamanna *et al* (1946) and Abrams *et al* (1946) and Type B was obtained in a highly purified but amorphous condition by Lamanna and Glassman (1947), while an improved procedure was described by Duff *et al* (1957) Type D toxin appears to be more lethal for mice than A or B because *unpurified* filtrates of cultures grown in cellophane (Sterne and Wentzel 1950) showed a toxicity little less than that of crystalline A toxin A purified Type D preparation (Wentzel *et al* 1950) showed potentiation in acid gelatin phosphate to about 20 000 times the toxicity of Type A toxin It would be most interesting to obtain confirmation of this remarkable but as yet isolated observation

The molecular structure of botulinum toxins appears to be unstable Purified Type A toxin was shown to be a protein of a molecular weight of about 1 000 000 (Putnam *et al* 1948) but Wagman and Bateman (1951 1953, Wagman, 1954) have shown that under certain conditions, the molecule may dissociate into fragments of molecular weight of approximately 70 000 some of which are inert and others more toxic than the original molecule Types B and D also seem to occur in forms with different molecular weights (Lamanna and Glassman 1947 Wentzel *et al*, 1950 Wagman and Bateman 1951 Duff *et al* 1957) Indeed Wagman (1963) has recently obtained *dialyzable* specific toxic fragments of botulinum toxin Thus botulinum toxin molecules do not appear to be fixed entities but structures capable of dissociating and repolymerizing in various ways Such changes may account for differences in the behavior of various types and preparations and possibly also for the ability of the toxin to pass from the gut into the circulation Indeed Type E toxin must be activated by proteolytic enzymes to reveal its full toxicity (Duff *et al* 1956)

In addition to the neurotoxins *C botulinum* also produces small amounts of a hemagglutinin an O labile hemolysin and a hemolytic lecithinase (see Wright 1955)

Pathogenesis

The disease is entirely toxemic and can be exactly simulated by parenteral or oral administration of the isolated toxin although it must be remembered that purification separates toxin from factors promoting gut permeability (Coleman 1954) It is one of the few toxins (and the only one of such formidable potency) that is not destroyed and indeed may be activated by the acid conditions and by proteolytic enzymes in the gut (Boroff *et al* 1952 Duff *et al*, 1956) It is unstable in the alkaline conditions prevailing in the greater part of the small intestine and, although it can survive the action of proteolytic enzymes in vivo Halliwell (1954) observed that it could be inactivated by several proteolytic enzymes in vitro but that no proteolytic digestion took place during the inactivation The toxin can be absorbed through the respiratory mucous membranes (an additional hazard to laboratory personnel working with dried toxin) as well as the gut walls The fact that the gut wall is slightly permeable to large molecules has been known for some time (Bullen and Batty 1956 Heckley *et al* 1960) and the special ability of botulinum toxin to act *per os* probably depends on its resistance to the acid of the stomach and on its great toxicity which enables the little that passes through the wall to kill The disaggregation already discussed may also play a part After absorption from the gut toxin can be found in the blood whence it is presumably absorbed by the peripheral nervous system

Prophylaxis and Treatment

Since the toxins of the different types of *C botulinum* are serologically distinct antisera that are used prophylactically should either be specific or be polyvalent to the extent of containing antibodies to A B and E the types to which man is naturally susceptible Antisera should be given to all persons suspected of having partaken of contaminated food Since the risk from botulism is ordinarily so slight there is no reason for active immunization with toxoid on a large scale although it is advisable to protect laboratory workers at special risk

Treatment with antitoxin has proved to be of little use in Type A intoxications probably because of the virtual irreversibility of the combination of toxin with nerve tissue and possibly because too little serum of too poor a quality was used It is more difficult to assess the value of serum in Types B and F

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24

The Spirochetes

INTRODUCTION

The order *Spirochaetales* includes diverse groups of spiral and actively motile micro organisms the majority of which divide by transverse fission Classification of the spirochetes has been principally on morphologic grounds and within each subgroup there are both pathogenic and nonpathogenic species There has been a tendency to attribute rela tionships among these diverse groups merely because of their spiral form when in fact other and perhaps more important biologic rela tionships are lacking As is the case in many other groups of bacteria the particular bio logic properties that confer to one species and not to another the ability to produce disease in man or animals are often obscure how ever it is probable that the active motility of these organisms plays a significant role in their disease producing proclivities The spirochetes historically and still today ac count for a substantial segment of human disease

The *Spirochaetales* have been divided into 2 families and 6 genera The family *Spiro chaetaceae* comprises 3 genera nonpatho genic for man *Spirochaeta Saprospira* and *Cristispira* All are large flexible and un dulating spiral organisms measuring from 30 to 500 μ in length the genera are distin guished by certain morphologic characteris tics *Spirochaeta* are found principally in sewage and contaminated water *Saprospira* in mud and sand and *Cristispira* in oysters and other molluscs All grow best at approxi mately 20 C

The *Treponemataceae* comprise 3 genera each of which contains species pathogenic for man the *Treponema* to which belong the causative organisms of syphilis yaws pinta and the other treponematoses the *Borrelia* which comprises the large group of relapsing fever spirochetes and the *Leptospira* to which belong various species causing lepto spirosis It is well to keep these 3 large groups of pathogenic spirochetes clearly separated for they have little in common except a spiral form which however is distinctive for each genus Interesting speculations concerning the evolutionary development of the spiral microorganisms have been made by Cock burn (1961) but conclusive evidence is lacking It should be noted too that each group contains species that are pathogenic for man others that are pathogenic for ani mals but not for man and still others that appear to be only saprophytic for man or animals In general their pathogenicity is not indicated by any morphologic characteristic and usually can be determined only by ani mal inoculation

The foregoing classification while useful and biologically justifiable has not been adopted universally and much confusion has been caused by the use of the term spiro- chete and the generic classification *Spiro chaeta* for any spiral organism For example the terms *Spirochaeta pallida* and *Treponema pallidum* are used almost interchangeably

Borrelia recurrentis is frequently referred to as *Spirochaeta recurrentis* and even at times as *Treponema recurrentis* or *Spironema recurrentis*

TREPONEMA AND THE TREPONEMATOSES

The treponematoses are a group of clinical and epidemiologic syndromes which have many features in common including (1) etiologic agent belonging to the *Treponema group of spirochetes* (2) subacute and chronic course with intervals of clinical quiescence and relapse (3) lesions of the skin and the bones (4) presence of the same type of serum antibodies (5) prompt response to penicillin therapy and (6) occurrence predominantly among individuals living under poor hygienic conditions

The treponema are slender spiral organisms which are readily distinguishable on morphologic grounds from the borrelia and the leptospira the morphologic characteristics will be described under *Treponema pallidum* which is the type species of this genera A good working classification is the following, although this arrangement has no standing in more formal taxonomic circles

1 **Human Pathogens Primarily**

A *T pallidum* The causative agent of syphilis

B *T pertenue* The causative agent of yaws

C *T carateum* The causative agent of pinta

D Treponemes that cause other human treponematoses such as bejel and other nonvenereal syndromes

2 **Animal Pathogens Primarily**

T cuniculi The causative agent of rabbit syphilis

3 **Human or Animal Saprophytes Primarily** Included in this group are spirochetes showing the general morphology of treponemes occurring in the oral cavity about the gum margins and about the anus and in fecal material The designations *T microdentium* and *T macrodentium* depending on their size have been applied to these organisms In contradistinction to those in categories 1 and 2 above these organisms can be cultivated on artificial media and a number of strains so cultivated have been given distinguishing names such as the Reiter the Noguchi the Kazan and the Nichols strains

Since syphilis and its causative organism, *T pallidum* have been the most extensively studied the fundamental biology of this disease will be described at some length In considering the other treponemal syndromes particular attention will be directed to pointing out either differences between them and syphilis or limitations in our knowledge of that particular entity

The question of the biologic relationship within the *Treponema* group of spirochetes has been studied by Turner and his associates (*Turner and Hollander, 1957*) among others and will be summarized briefly in the final section of Treponema and the Treponematoses

TREPONEMA PALLIDUM AND SYPHILIS

History

Syphilis became epidemic in western Europe in 1492 and the immediately succeeding years It is generally believed to have been acquired by Columbus crew in the West Indies and brought by them to Europe but only meager evidence supports this hypothesis It appears more likely that syphilis and related treponematoses were endemic in Africa and Europe centuries before and reached epidemic proportions during the mass movements of armies and populations at the end of the 15th and the beginning of the 16th centuries The extraordinary virulence of the disease at that time appears clearly in some of the early descriptions It is not known whether the present relatively mild course of the disease in its early stages reflects a change in the organism or a gradual development of resistance in the human host The causative organism was identified in 1905 when Schaudinn and Hoffman demonstrated its presence in the primary lesion and in the adjacent lymph glands of syphilitic patients Noguchi and Moore subsequently found the organisms in the cerebral cortex of patients dying with general paresis thus they proved that syndrome to



Fig 1 (*Top*) Congenital syphilis of the lung *Treponema pallidum* demonstrated by Levaditi s method (Smith L W and Gault E S 1942 Essentials of Pathology ed 2 New York Appleton)

Fig 2 (*Bottom*) *T pertenue* of the skin secondary yaw Oil immersion photomicrograph stained by Levaditi s method The organisms appear as irregularly twisted spirals lacking the tight corkscrew appearance of the *Treponema pallidum* (Smith L W and Gault E S 1942 Essentials of Pathology ed 2 New York Appleton)

be as had long been suspected a late manifestation of syphilitic infection

Characteristics of the Treponeme

Morphology *Treponema pallidum* is a fine spiral organism measuring 5 to 20 microns in length and about 0 1 to 0 2 micron in thickness it is difficult to see in the ordinary light microscope hence the derivation of its present name—a pale fine thread When suspended in serum the spirals are regular and angular from 4 to 14 in number with the depths of the spirals being from 0 5 to 1 micron and the distance between them about 1 micron In this medium the organism has a fairly rapid rotary motion with little or no translation and while difficult to describe both its morphology and its type of movement are distinctive and serve to differentiate the treponema from the borrelia and the leptospira

However in a more viscid medium such as the mucoid material of the early syphilitic lesion the organism may have a more elongated appearance as though a coiled spring were overstretched and its motion may be writhing undulating and snakelike in character with considerable movement of translation Both morphology and motility can be altered artificially by changing the density of the medium

As demonstrated by electron microscopy *T pallidum* in common with most spiral microorganisms has an axial filament about which the specially shaped protoplasm is wound and the protoplasm is encased in a thin periplast Winding around the protoplasm probably within the periplast are a number of fibrils which usually run the entire length of the structure (Bradfield and Cater 1952 Ryter and Pillot 1963 Swain 1955 Molbert 1956) Some investigators have demonstrated flagellalike tufts at the ends or the sides of treponemes (Wile and Kearney 1943 Mudd Polevitzky and Anderson 1943) but studies of cultivable spirochetes suggest that these flagella may be the above mentioned fibrils from treponemes in process of degeneration (Listgarten Loesche and Socransky 1963)

The presence of capsular material probably mucoid in character has been postulated largely on the basis of indirect evidence rather than by visualization (Swain, 1955 Turner and Hollander 1957) A mucopolysaccharide splitting enzyme lysozyme has been found to accelerate immobilization of *T pallidum* by specific immune serum (Metzger Hardy and Nell 1961)

Treponemes stain readily with many different dyes but because the mass of protoplasm is so slight contrast is not achieved therefore from a practical point of view staining methods are unsatisfactory (Campbell and Rosahn 1950) The silver staining method of Levaditi with its many modifica

tions is based on the deposition of metallic silver on the surface of the treponeme thereby increasing the contrast Due to irregular deposition of silver in fixed tissue sections this stain gives variable and unpredictable results

Cultivation *T pallidum* and other pathogenic species of treponemes have not been grown in vitro despite isolated reports from time to time of successful cultivation Indirect evidence suggests that the pathogenic treponemes are obligatory anaerobes Attempts to grow the pathogenic organism in fertile eggs or in tissue culture have failed (See Cultivable Treponemes)

Resistance to Physical and Chemical Agents The survival time of *T pallidum* in vitro is affected by a number of factors Suspensions of organisms adequately freed of serum and tissue extractives and resuspended under anaerobic conditions in a fluid containing crystallized serum albumin dissolved CO either cysteine or glutathione pyruvic acid and a serum ultrafiltrate factor, may remain actively motile for 4 to 7 days at 25 C and for 1 to 2 days at 37 C (Nelson 1948 Rice and Nelson, 1951) Although supplementation with a number of additional factors further prolongs survival there has been no evidence of multiplication (Weber 1960)

When frozen at the temperature of dry ice (approximately −70 C) a significant proportion of the organisms remains motile and infectious for years (Turner and Fleming 1939 Turner and Hollander, 1957) The proportion of treponemes that survive the freezing and thawing process is considerably enhanced by the addition of 15 per cent glycerol to the suspending medium (Hollander and Nell, 1954) In plasma whole blood or serum stored at refrigerator temperatures the organisms remain viable for 24 hours but not for 48 (Ravitch and Chambers 1942), a fact of importance in relation to the problem of transfusion syphilis The organisms do not survive desiccation by freezing and drying technics customarily employed in the preservation of viruses However, Hampp (1951) reported survival for as long as 66 days in desiccated minced testicular syphilomas from rabbits In the tissues after death, the treponemes may remain infectious for 1 to 5 days In infected animals *T pallidum* apparently may be killed by elevating the body temperature to 41 5 to 42° C and the treponemicidal action of both the arsenicals and penicillin in vivo is enhanced at

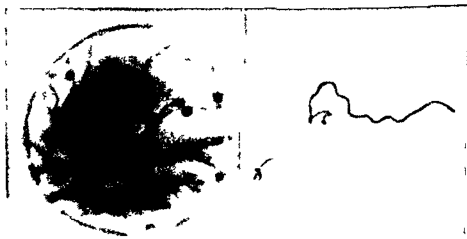


Fig 3 (*Left*) Kazan strain of cultured spirochete Multispirochetal cyst showing development of spirochetes from individual granules (*Right*) Nichols strain of cultured spirochete Late stage in emergence from unispirochetal cyst (Delamater E D *et al* Am J Syph *35* 164 216)

those higher temperatures Whether the demonstrated therapeutic action of malaria in cases of neurosyphilis is due solely to the treponemicidal action of the higher temperatures whether the body s natural defense mechanisms are enhanced at those higher temperatures and whether antibodies are elaborated in the course of malarial infection which cross react with *T pallidum* remain open questions

The organisms are immobilized rapidly by trivalent arsenicals bismuth and mercurials There is reason to believe that these compounds are treponemicidal by virtue of their common affinity for –SH groups in the organism and probably because they block essential –SH groups in enzyme proteins vital to the cellular economy

Penicillin is directly treponemicidal both for the pathogenic *T pallidum* and for the cultivated saprophytes *T pallidum* is one of the most sensitive organisms yet studied in terms of the minimal effective concentrations of penicillin both in vivo and in vitro Paradoxically it is also one of the most resistant organisms in terms of the rate of the treponemicidal effect (Eagle Fleischman and Musselman 1950)

Cultivable Treponemes Oral spirochetes were first cultivated on artificial media by Muhlens and Hartman in 1906 and by numerous investigators since that period (see review of literature in Hardy Lee and Nell 1963a) Many of these organisms may be classified morphologically as treponemes Cultivable treponemes are consistently nonpathogenic for experimental animals and presumably for man except for surface infection of mucous membranes They are generally regarded as being not closely related to pathogenic treponemes However they do possess certain antigenic fractions in common with virulent *T pallidum* (D Alessandro and Dardanoni 1953)

The Reiter strain has been especially well studied largely in the hope that leads would be obtained concerning cultivation of virulent *T pallidum* although in the latter connection the results thus far have been disappointing It is clear too that requirements differ somewhat from one species of cultivable spirochete to another

Good growth of the Reiter strain is obtained in anaerobic broth or thioglycollate media provided that serum is added more detailed requirements have been delineated by Steinman and Eagle (1950) and Hardy Lee and Nell (1963a) among others The latter investigators have also reported the growth of numerous strains on solid media and find consistent differences in colonial morphology which may have taxonomic significance Using media with low agar concentration (0 7%) both surface and deep colonies were observed and stable colonial variants were produced Some strains were hemolytic In certain types of colonies the spirochetes presented variable and often bizarre forms some of which were not spiral while in other types of colonies the spirochetes commonly presented the characteristic spiral form observed in broth cultures

Many cultivable spirochetes have been isolated as mixed cultures Hardy Lee and Nell (1963b) found that certain mouth spirochetes would grow on artificial media only in the presence of contaminating bacteria isolated usually from the oral cavity of the same person Further a filtrate of some of these bacteria produced a similar enhancing effect

Various developmental phases in the growth of the Reiter and other nonpathogenic spirochetes have been observed (Gelperin 1949 Hampp 1951 DeLamater Haanes Wiggall and Pillsbury 1951) Gelperin found that when this organism is maintained in culture for 2 or 3 weeks under adverse conditions balloonlike transparent spheres which contain small round translucent bodies appear at one end of nonmotile spirochetes It has been sugge ted that these balloons represent an encystment stage but Hardy and Nell (1961) observed that these changes could be induced by alteration of the osmotic pressure of the media and tended to view the spheres as simply degenerative forms of the spirochete

Host Range and Pathogenesis

T pallidum is the etiologic agent of syphilis and in nature is confined to its human host It has proved to be infectious for rabbits and monkeys in which it causes infections which resemble the human disease in

tions is based on the deposition of metallic silver on the surface of the treponeme, thereby increasing the contrast Due to irregular deposition of silver in fixed tissue sections this stain gives variable and unpredictable results

Cultivation *T pallidum* and other pathogenic species of treponemes have not been grown in vitro despite isolated reports from time to time of successful cultivation Indirect evidence suggests that the pathogenic treponemes are obligatory anaerobes Attempts to grow the pathogenic organism in fertile eggs or in tissue culture have failed (See Cultivable Treponemes)

Resistance to Physical and Chemical Agents The survival time of *T pallidum* in vitro is affected by a number of factors Suspensions of organisms adequately freed of serum and tissue extractives and resuspended under anaerobic conditions in a fluid containing crystallized serum albumin dissolved CO either cysteine or glutathione pyruvic acid and a serum ultrafiltrate factor may remain actively motile for 4 to 7 days at 25 C and for 1 to 2 days at 37 C (Nelson 1948 Rice and Nelson, 1951) Although supplementation with a number of additional factors further prolongs survival there has been no evidence of multiplication (Weber 1960)

When frozen at the temperature of dry ice (approximately −70° C) a significant proportion of the organisms remains motile and infectious for years (Turner and Fleming 1939 Turner and Hollander 1957) The proportion of treponemes that survive the freezing and thawing process is considerably enhanced by the addition of 15 per cent glycerol to the suspending medium (Hollander and Nell 1954) In plasma whole blood or serum stored at refrigerator temperatures the organisms remain viable for 24 hours but not for 48 (Ravitch and Chambers 1942), a fact of importance in relation to the problem of transfusion syphilis The organisms do not survive desiccation by freezing and drying technics customarily employed in the preservation of viruses However, Hampp (1951) reported survival for as long as 66 days in desiccated minced testicular syphilomas from rabbits In the tissues after death the treponemes may remain infectious for 1 to 5 days In infected animals *T pallidum* apparently may be killed by elevating the body temperature to 41 5° to 42 C and the treponemicidal action of both the arsenicals and penicillin in vivo is enhanced at

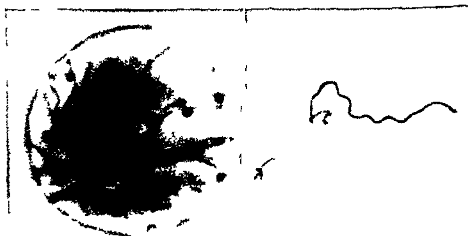


Fig 3 (*Left*) Kazan strain of cultured spirochete Multispirochetal cyst showing development of spirochetes from individual granules (*Right*) Nichols strain of cultured spirochete Late stage in emergence from unispirochetal cyst (Delamater E D, *et al* Am J Syph *35* 164 216)

proportion to the number of organisms demonstrable either by darkfield examination or by animal inoculation strongly suggesting that the tissues have become sensitized to the products of the organisms

In rabbits the disease in its initial stage differs in no important respect from the disease in man Animals may be infected via various routes including the eye the skin the testis or the scrotum and intravenously One spirochete (as statistically determined) inoculated intratesticularly is infectious and 4 organisms inoculated intradermally cause infection in half the animals (Magnuson Eagle and Fleischman 1948) The time required for the development of a macroscopic inflammatory lesion at the site of inoculation varies with the size of the inoculum with an average linear decrement of 4 days for each 10-fold increase in the number of spirochetes inoculated in the range 1 to 1 million Inoculation of 500 organisms intradermally yields an incubation period of approximately 17 days This suggests a division time in vivo of approximately 30 hours (Magnuson Eagle and Fleischman 1948 Cumberland and Turner 1949 Hollander Turner and Nell 1952) The slow rate at which the organisms multiply in vivo in both man and rabbit is further indicated by the months which may elapse between the completion of inadequate treatment for early syphilis and the appearance of relapsing lesions

There are conflicting reports concerning the speed with which the organism leaves the site of inoculation and invades the regional lymph nodes and the circulation in the experimental infection but most observations place the time as a matter of minutes or hours The rate of migration probably varies with the animal species the particular tissue involved its vascularity and lymphatic drainage and the degree of tissue damage incidental to the inoculation

The rabbit infection differs from that of man in several important respects In man a certain proportion of the cases apparently undergoes spontaneous cure in a biologic sense and the lymph nodes even in untreated patients remain infectious in only a small number of instances However the rabbit apparently remains infected for the rest of its natural life Organisms persist in lymph nodes the spleen and bone marrow and can be demonstrated by inoculating those tissues into normal animals Frazier and his co-workers have shown that transient spirochetemia occurs in infected rabbits for a number of years after all overt signs of infection have disappeared—and possibly at intervals throughout the animal s life (Frazier Bensel and Keuper 1950 1952) In rabbits once the early stages of the disease have healed there is only a minor microscopic inflammatory reaction even in tissues known to harbor the organisms Although skin bone and the eyes may be involved in the early inflammatory process late visceral manifestations (heart liver and central nervous system) have been described only rarely

Monkeys and chimpanzees are susceptible to inoculation with *T pallidum* (Metchnikoff and Roux 1903) The early course of the disease parallels that in man and in the rabbit but it is not known whether the animals develop late complications resembling those seen in the human infection Mice and rats were shown by Kolle and Schlossberger (1926) to harbor the organisms for long periods without developing gross lesions and a variety of rodents have been found to undergo a similar asymptomatic infection (Bessemans and de Moor 1939 Wile and Johnson 1945) A small proportion of infected hamsters and guinea pigs develops a lesion at the site of inoculation of *T pallidum* (Turner and Hollander 1957)

Evolution of the experimental disease is influenced by a number of factors aside from those associated with specific immunity Among the most important of these so-called nonspecific factors are trauma temperature and certain hormonal influences

In both experimental and human infections *T pallidum* has been shown to have a special predilection for inflamed or traumatized areas the underlying mechanisms involved are not understood (Chesney Turner and Halley 1928)

There is considerable indirect evidence that in rabbits *T pallidum* multiplies best at a temperature approximating 35 C rather than at body temperature which is normally 38 C or slightly higher In rabbits

many respects but with important differences In mice and many other rodents, the organisms multiply only poorly without producing a significant tissue reaction but are demonstrable by subinoculation into rabbits No mammalian species has been found to be wholly resistant to infection with *T pallidum*

The human infection is usually transmitted by sexual contact In men the organisms are often present in lesions on the penis but they may originate anywhere in the genitourinary tract and be discharged with the seminal fluid In women organisms may derive from mucocutaneous perineal lesions cervical lesions or mucous patches on the vaginal wall In approximately 10 per cent of cases the infection may be extragenital usually caused by a chancre or a mucous patch on the lip or the tonsil It is doubtful that the organisms can penetrate the intact skin however it is possible that they can penetrate the thinner epidermal layer of the mucous membranes and it is probable that in many persons they gain access through a break perhaps only microscopic in the epidermal layer Although some of the organisms move away from the site of inoculation to reach the adjacent lymph nodes and thence cause a systemic infection within a period of hours to days most apparently remain at the site of infection

Specific tissue changes characterized by accumulations of mononuclear cells around blood vessels and at foci of treponemes begin within 48 hours after injection of treponemes (Ferris and Turner 1938) In the experimental infection the rapidity with which these tissue changes develop is a direct function of the number of treponemes in the original inoculum (Cumberland and Turner 1949), and this is probably also true of the human infection Eventually a characteristic inflammatory response known as the primary lesion or chancre becomes clinically recognizable within 10 to 60 days This lesion—wherever it is located—is firm and often quite hard due to the presence of intense cellular infiltration and mucoid and serum accumulations in connective tissue cells hence the old clinical designation hard chancre to distinguish it from lesions due to certain bacterial infections—especially *B ducreyi*, which gives rise to chancroid or so-called soft chancre

An almost constant accompaniment of the initial lesion of syphilis and an important diagnostic sign is significant enlargement of the lymph node draining the local area This 'satellite bubo is characteristically firm and painless From 2 to 12 weeks later a generalized skin rash appears in most patients, with treponemes demonstrable in the lesions This rash exhibits lesions of varying size morphology and distribution and may be so slight and evanescent as to go unnoticed by the infected individual When syphilis was first recognized around the turn of the 16th century this rash was so extensive and severe that it received the appellation of the Great Pox as distinguished from smallpox and even within the present generation an occasional case of this nature has been observed

In this secondary stage syphilitic lesions may involve the mucous membranes the eyes the bones and the joints and the central nervous system reflecting generalized dissemination of organisms and their multiplication at countless foci

The evolution and the extent of the generalized lesions represent an interplay between host and parasite specific antibodies begin to develop and the immunity mechanism of the host comes into play Even in the absence of specific treatment the generalized lesions usually heal and the disease enters a stage of clinical latency

The subsequent course of the disease is extraordinarily varied Presumably in most untreated persons foci of treponemes remain although the studies of Bruusgaard (1929) Gjestland (1955) Clark and Danbolt (1955) and others indicate that approximately 25 per cent of infected persons proceed to apparent spontaneous biologic cure and an equal proportion never again have symptoms referable to the disease even without specific treatment Approximately half of those infected develop late complications varying in severity and prognosis from the relatively benign gummas of the skin and the bone to the prognostically serious cardiovascular or central nervous system involvement In the late skin lesions the intensity of the cellular reaction is out of all

immunity is interrupted and remains feeble On the other hand when curative treatment is delayed until the third month immunity to challenge inoculation is maintained at a high level for months and even years

Serum Antibodies Concomitant with the early evolution of the initial syphilitic lesion the experimental animal (and man too) develops serum antibodies which may be regarded as specific for treponemal infections although some qualification of this statement will appear below These antibodies are of several kinds and in the light of limited knowledge appear to be induced by antigenic stimuli provided by the infecting treponeme Serum antibodies in syphilis and the serologic tests by which these antibodies are demonstrated can be divided from a biologic as well as a historical standpoint into 2 large groups which may be designated for lack of better terms Wassermann antibody and treponemal antibody

Wassermann Antibody In 1906 Wassermann Neisser and Bruck using complement fixation technics developed by Bordet and Gengou (1901) discovered what has become known as Wassermann antibody Since fetal liver rich in *T pallidum* was the first antigen used in the Wassermann test the complement fixing antibody which is commonly present in high titers in patients with early syphilis was regarded as an antibody specific for that organism However soon it was discovered that this antibody could just as readily be demonstrated by using normal liver as antigen and later it was demonstrated that alcoholic extracts of a wide variety of human or animal tissue could serve as the antigen in the Wassermann test The antigen commonly used at present is a highly purified lipid extract of beef heart the so-called cardiolipin to which lecithin and cholesterol have been added (Pangborn 1945)

Much speculation and investigative work have been devoted to the question of whether this antibody regularly present in most human beings and animals which have had a treponemal disease is a true antibody to a component of the treponemal organisms or whether it represents a response to some abnormal tissue lipid component developed during the course of treponemal infection No attempt will be made here to summarize the evidence bearing on this question suffice it to say that the author inclines to the first point of view while equally well informed colleagues favor the second

At any rate for 35 years tests based on the demonstration of Wasserman antibody (or reagin as it is sometimes called) constituted the principal laboratory aid to the clinician in the diagnosis of syphilis and related diseases and indeed so they remain today During that period a large number of laboratory tests were developed for the serologic diagnosis of syphilis each usually being denoted by the name of the originator In general two kinds of tests were devised one being based on the principle of complement fixation as indicated above and another utilizing the principle of aggregation of particles in the presence of specific antibody or flocculation as it is generally known Originally this latter technic was developed by Michaelis (1907)

Thus in common use today are complement fixation tests designated Boerner Lukens Eagle Kolmer Wassermann and flocculation tests known as Davies Eagle Hinton Kahn, Kline Meinicke Muller Mazzini Rein Bossak VDRL and others (It is historically ironic that the names of Bordet and Gengou and of Michaelis have been almost wholly lost in these relationships) Since these tests represent modifications of the same basic principle and detect the same antibody it has become the practice in many places to include them all under the designation serologic tests for syphilis or S T S for short

However in the past few years there has been a growing awareness that some individuals who never have had syphilis or any other treponemal disease nevertheless have Wassermann antibody in significant amounts in their serum as demonstrated by positive complement fixation or flocculation tests for syphilis Such transient biologic false positive serologic tests for syphilis are not uncommon during the course of many acute illnesses or immediately following certain vaccination procedures More confusing from both a clinical and a biologic standpoint are individuals who carry significant amounts of Wassermann antibody in their

the incubation period of the initial lesion fol lowing inoculation of *T pallidum* into the shaved skin is shorter and the resulting lesions are much larger when the animal is maintained at a cool environmental tempera ture (18 to 21 C) than at a warm temperature (29 to 31 C) at the latter tem perature lesions develop poorly or not at all These differences apparently are not due to changes in the internal body temperature of the animal The same phenomenon is ob served in hamsters In both these species there are indications that localization of syphilitic lesions may be influenced consider ably by local tissue temperature in rabbits generalized lesions of skin and bone occur principally on the extremities and about the nose the ears and the tail where the local temperature may be 1 to 3 lower than in ternal body temperature (Bessemans, 1938 Hollander and Turner 1954)

Hormonal factors likewise have been shown to be influential in experimental in fections of the rabbit Estrogens tend to make the disease milder and androgens more extensive (Kemp Shaw and Fitzgerald 1939 Hu 1939) However the most dra matic effect is produced by the administra tion of cortisone Given during the early phases of the disease cortisone in doses of 3 to 6 mg per Kg of body weight per day causes the syphiloma to become softer and more mucoid and to contain tremendous numbers of motile treponemes Withdrawal of cortisone usually results in a rebound phenomenon in which the syphilitic lesions increase rapidly in number and size to produce extensive disease These changes appear to be due primarily not to the effect of cortisone on the production of antibody but to some alteration in the local tissue parasite relationship It is postulated that the treponeme itself is the source of the great overproduction of mucoid material which has been identified as being mainly hyaluronic acid (Turner and Hollander 1952) To what extent these phenomena occur in human infection is not known

Immunity Phenomena in Syphilis

Once a patient has been through the stages of early syphilis he is unlikely again to have primary or secondary syphilis even in the face of repeated exposure to infection Reinfection in syphilis may occur, but it is usually symptomless except in patients who were given specific treatment in the early stages of the first infection Clinical and ex perimental observations indicate that these results can be explained on an immunologic basis

For many years it was believed that im munity in syphilis because of its slow de velopment and its limited effectiveness was qualitatively different from the immunity mechanisms involved in most other infec tious processes The extensive studies of Chesney Uhlenhuth Tani Magnuson Tur ner Nelson and Hardy and their respective co workers provide a body of evidence sup porting the conclusion that immunity in syphilis is qualitatively similar to that in most other infections although to be sure knowledge in many areas is deficient es pecially does the whole phenomenon of latency in treponemal infection—and in deed in many other infections—remain obscure as to its basic mechanism

Host Reaction In experimental syphilis in the rabbit beginning resistance to a second infection becomes evident about 3 weeks after the appearance of the initial lesion although the rapidity of development and the degree of immunity is clearly related to the number of treponemes *in the host* (and probably the antigenic mass) as mani fested by the number and the extent of the initial lesions The evolution of the immune process may be interrupted at any time by penicillin treatment of the animal Asympto matic syphilitic infection is not capable of inducing immunity but once immunity is fully developed latent infection may play a role in its maintenance (Magnuson Thompson and Rosenau 1950 Hollander Turner and Nell 1952)

In the normal evolution of the experi mental disease resistance to challenge inocu lation reaches its height 2 to 3 months after the beginning of the first infection In the absence of specific treatment it is then main tained at a high level for the remainder of the animal s life Contrary to the course of immunologic events in untreated syphilis when curative treatment is given early dur ing the first infection the development of

1955) and particulate adhesion of the Rickenberg type (Lamanna and Hollander 1956) None of these technics has been subjected to sufficient clinical evaluation to permit assessment of their clinical or biologic merit although in general the results of these tests show a close parallelism with those of the treponemal immobilization test However there are indications as pointed out below that these tests may not be measuring the same antibody even though ordinarily the antibodies in question may occur together in nature

More recently fluorescent antibody technics utilizing methods originally developed by Coons and his co-workers (Coons and Kaplan 1950 Coons 1954 1960) have been applied as a diagnostic aid in syphilis While their clinical role has not yet been clearly evaluated these so-called FTA tests appear to have some promise *T pallidum* harvested from rabbits testes used fresh or from the frozen state or the frozen and dried state are customarily employed as antigen After appropriate fixing the human serum to be tested is brought into contact with the antigen followed by fluorescein labeled antibody to human serum Results of the clinical use of the test have been reported by Deacon and his associates and by Neilson and Idsoe among others (Deacon and Freeman 1960 Neilson H A and Idsoe O 1963) Considerable cross reactions between pathogenic *T pallidum* and the cultivable varieties have been reported (Deacon and Hunter 1962) which may limit the value of this test in diagnostic serology

Antigenic Fractions of Treponemes Reflecting a somewhat different approach application of modern immunochemical methods to the study of fractions of treponemes has revealed the antigenic complexity of these organisms it is probable that knowledge in this area will be extended considerably in the next few years For example Hardy and Nell (1957) working with agglutination technics have described both a heat labile and a heat stable fraction of pathogenic treponemes each of which is capable of inducing its own specific antibody which appears to differ in specificity from the other and from both immobilizing and Wassermann antibody Likewise Portnoy and Magnuson (1955) have prepared by desoxycholate extraction fractions of virulent *T pallidum* which react specifically with syphilitic serum The antibody thus detected appears to parallel fairly closely the occurrence both of immobilizing and of agglutinating antibody but its exact relationship to these has not been determined Still another area of investigation which may prove to be of practical as well as theoretical value has been pursued by D Alessandro and Dardanoni (1953) These investigators have found 4 serologically active components of the nonpathogenic Reiter cultivable treponeme One of the components was considered to be a soluble protein and this fraction has been used in the Reiter Protein Complement Fixation Test (RPCF) However it appears that by current methods of extraction various batches of antigen vary in their chemical and antigenic composition (Cannefax 1963) and as pointed out by DeBruijn (1962) and Bekker (1962) there are inherent limitations in the use of this test as indeed in the use of all serologic tests for syphilis

However it should be noted that although the repeated injection of rabbits with whole killed *T pallidum* or one of its fractions and with large quantities of the Reiter cultivable treponeme or one of its fractions may give rise to a variety of antibodies including Wassermann antibody depending on how the treponeme suspension has been treated it has not yet been possible to induce the development of treponemal immobilizing antibodies except in very low titer This leads to the speculation that both specific immunity and immobilizing antibody are induced by some highly labile antigenic component or components of pathogenic treponemes a fraction possibly associated with the mucoid slime layer of the organism

Suspensions of killed washed and concentrated *T pallidum* have also been used in an intradermal test after the fashion of tuberculin (Marshak and Rothman 1951 Csonka, 1955) Positive tests are observed most commonly in patients with late syphilis but neither the immunologic nor the clinical significance of these observations has been demonstrated conclusively

Antibodies in Other Tissues Antibodies in syphilis are like other antibodies associ

serum over periods of months or years and yet according to convincing evidence never have had a treponemal infection Some evidence has been adduced by Moore and Mohr (1952) that the presence of such antibody may indicate the existence of one of the collagen diseases in either an overt or an asymptomatic form The possibility that concurrent infection with spirochetes of the Reiter group may produce such antibodies has never been definitively explored (However see Deacon and Hunter 1962)

Treponemal Antibodies When syphilitic serum is mixed with suspensions of viable *T pallidum* and these mixtures are then inoculated into rabbits the production of lesions is either delayed or prevented (Tani and Ogiuti 1936 Turner 1939 Turner *et al* 1948) Further when tissue containing *T pallidum* is implanted into the skin of an immune rabbit there is progressive death of the organisms in the implant treponemes are not demonstrable in the adjacent lymph nodes and the implant loses its infectivity after 2 days However, in normal animals the treponemes readily penetrate into the lymphatics to cause generalized infection and remain viable in the implant for at least 2 weeks (Tani and Aikawa 1937 Reynolds 1941)

In 1949 Nelson and Mayer produced convincing evidence of the existence in human beings and animals with syphilis of an antibody that reacts specifically with *T pallidum* and closely related treponemes and is separate and distinct from Wassermann antibody Demonstration of this antibody was accomplished through immobilization of *T pallidum* in the presence of complement and has since become known as the Treponemal Immobilization Test or TPI test Its discovery was facilitated by the earlier development by Nelson (see p 576) of methods for the prolonged maintenance of pathogenic treponemes *in vitro* Simple in principle but difficult of execution the TPI test utilizes a concentrated suspension of motile *T pallidum* extracted from syphilomas of rabbits testes the human or animal serum to be tested and fresh guinea pig serum containing complement An appropriate mixture of these materials is incubated anaerobically for 18 hours and the test is read by determining under the darkfield microscope the proportion of treponemes which have been immobilized (i e which have lost motility and presumably, have been killed) as contrasted with the proportion still motile in tubes containing known normal serum Syphilitic serum repeatedly absorbed with cardiolipin antigen until it no longer contains demonstrable Wassermann antibody will continue to immobilize treponemes in essentially undiminished titer

Although technical difficulties have limited the wide use of the TPI test in practice a large number of clinical and laboratory studies have demonstrated its high degree of reliability in reflecting the presence or the absence of prior infection with *T pallidum* or closely related treponemes

During the course of the natural infection in man and the experimental infection in rabbits immobilizing antibody along with Wassermann antibody can be detected in the blood serum about 1 to 3 weeks after the appearance of the primary syphilitic lesion The titer of immobilizing antibody continues to rise over the next few weeks or months and remains at a high level in untreated persons and animals for many years and probably for life There are some data suggesting that immobilizing antibody tends roughly to parallel immunity to reinfection in syphilis but the evidence on this point is by no means conclusive However it is clear that the titer of Wassermann antibody at least in the experimental animal does not parallel the immune state but rather is correlated with the number and the extent of active lesions For example rabbits 6 months after initial infection commonly show no evidence of active disease and Wassermann antibody is low in titer or undetectable yet these same animals almost invariably show a high degree of immunity to challenge inoculation and immobilizing antibodies in high titer

Demonstration of immobilizing antibody stimulated the application of other technics for the detention of this or related antibodies Among such technics described have been immune adherence (Nelson 1953) treponemal agglutination (McLeod and Magnuson, 1953 Hardy and Nell 1955) complement fixation (Portnoy and Magnuson

specimens for evidence of treponemal disease

At the present time tests for treponemal antibody—TPI agglutination complement fixation—are of greatest practical value as an aid in identifying patients with biologic false positive tests for Wassermann antibody Persons who show positive tests for Wassermann antibody in the absence of clinical or epidemiologic evidence suggesting syphilis should have their serum tested for the presence of treponemal antibody before a diagnosis of syphilis or other treponemal disease is established

Serologic tests have been used traditionally as a guide to therapy in syphilis and related diseases but their interpretation is fraught with difficulty It is a moot question whether antibody production continues for long after the antigenic stimulus provid d by the treponeme has been eliminated Data from experimental animals with well-established infections indicate that following curative treatment Wassermann antibody declines fairly rapidly but treponemal antibody remains present for long periods although declining gradually in titer

There is some evidence suggesting that the same phenomena occur in human beings on an extended time scale but the data are not conclusive Following successful treatment of early syphilis both Wassermann antibody and treponemal antibody decline over a period of months the former more rapidly than the latter so that eventually most of such patients show negative serologic tests In more long standing cases the decline in both types of antibody is much more gradual in many patients tests for Wassermann antibody eventually become negative whereas in most individuals tests for treponemal antibody remain positive It can be argued that the persistence of these antibodies reflects persistence of infection but clinical and immunologic evidence does not indicate that this is necessarily true There is much indirect evidence suggesting that, just as tetanus toxoid induces specific antibodies that persist for many years both Wassermann and treponemal antibody also may persist for many years after elimination of all treponemes by penicillin therapy

Therefore in the present state of our knowledge it would be unwise to assume that individuals who have received what is regarded as adequate treatment for syphilis and still show the presence of these antibodies are still infected Perhaps the best serologic guide to adequate therapy is evidence of some decline in titer of these various antibodies recognizing that such a decline may be detectable only over a period of years and not necessarily over a period of weeks or months

Spinal Fluid Tests Under normal circumstances the serum antibody does not pass into the spinal fluid even in syphilitic patients with a high serum titer In patients with central nervous system syphilis the antibody appears in the cerebrospinal fluid either because of damage to the blood brain barrier or through local elaboration Therefore the presence of a positive test for Wassermann or treponemal antibody in the spinal fluid is usually indicative of involvement of the central nervous system The antibody titer of the fluid coupled with its cell content and protein content and their response to antisyphilitic treatment provide information of diagnostic and prognostic value Tests for treponemal antibody have had less thorough clinical evaluation than have tests for Wassermann antibody

Treatment

Until the discovery and the development of penicillin the arsenicals notably arsphenamine and its derivatives in conjunction with bismuth were the drugs of choice in the treatment of syphilis Now these have been entirely superseded by penicillin which has been shown to be far more active as a treponemicidal agent as well as much less toxic for the patient

Penicillin was first shown to be effective against *T pallidum* by Mahoney Arnold and Harris (1943) its use has revolutionized the therapy of syphilis and other treponemal diseases Instead of months and often years of therapy with arsenicals and heavy metals all of which were inherently toxic to the patient therapy is now accomplished in most patients within a period of 2 weeks although much longer periods of observation are desirable

ated with the globulin fraction of serum and may be found wherever serum globulins abound For example, such antibodies enter the fetal circulation and may be found in cord blood and in the infant for a number of months after birth These antibodies may also be found in the cerebrospinal fluid of syphilitic patients particularly if the blood brain barrier is sufficiently altered to permit easy passage of serum globulins Indirect evidence suggests that active syphilitic disease of the central nervous system may also induce the local formation of these anti bodies

Diagnosis

The diagnosis of syphilis in its numerous manifestations rests on (1) clinical observa tion (2) the demonstration of *T pallidum* usually by darkfield examination of the ex udate from an open or abraded primary or secondary lesion and (3) serologic changes in the blood and the spinal fluid

The diagnostic use of the darkfield ex amination is complicated by the fact that exudates from nonsyphilitic lesions may con tain spiral organisms Although most of these can be differentiated readily from *T pallidum* by their larger size coarser spirals and different type of motility a few may be difficult to distinguish with certainty On the *other hand a negative darkfield does not* necessarily exclude syphilitic infection In the late stages of the disease the number of spirochetes is extraordinarily small in rela tion to the degree of the inflammatory re action and the demonstration of treponemes whether by staining darkfield examination or even animal inoculation is rarely suc cessful Even in the early lesions failure to detect the organisms in the exudate does not exclude their presence in large numbers *Thus* if a drop measuring 0 01 ml is placed under a coverslip 22 mm square and ex amined at a magnification of 900× each field represents approximately 10^{-6} ml of fluid The presence of 1 organism per field then implies the presence of 10^6 organisms per ml but conversely, the absence of visi ble treponemes *in even 100 microscopic* fields is compatible with the presence of as many as 10 000 treponemes per ml of fluid Fortunately, the number of organisms in the exudate of early lesions is usually so large that 1 or 2 preparations suffice to de tect them, when few in numbers the organ isms usually can be found by repeated darkfield examinations of the exudate over a period of several days avoiding the use of local antiseptics

Serologic tests constitute the most fre quently used method by which a diagnosis of syphilis is made or confirmed Some of the problems and the limitations in the sero logic diagnosis of syphilis and related dis eases have been indicated in the foregoing section By and large, these tests when used singly or in combination do provide the clinician with reliable and valuable diagnos tic evidence however their use as guides in the therapeutic management of the patient have greater limitations

During the incubation period of syphilis and for the first 1 to 3 weeks after develop ment of the chancre or the initial lesion all serologic tests are commonly negative Thenceforth however with extremely few exceptions patients with clinically overt syphilitic disease show positive serologic tests for syphilis both those that detect Was sermann antibody and those that show the presence of so called specific treponemal antibody

Patients with latent syphilis i e no physi *cal signs of the disease* as a rule also have positive serologic tests indeed in the final analysis a diagnosis of latent syphilis is based solely on such tests However it is known that a small proportion of patients may have negative tests for Wassermann *antibody but positive tests for treponemal* antibody, it is in such cases that the TPI and related tests are valuable Because of the technical difficulties inherent in the TPI test it is not feasible at the present time to test for treponemal antibody all blood specimens which show a negative *test for* Wassermann antibody In practice therefore it is custom ary to test for treponemal antibody speci mens from patients in whom the presence of syphilis is suspected on clinical or epide miologic grounds Improvements in technics and further *evaluation of some* of the tests for treponemal antibody may eventually permit their substitution for the Wassermann tests in the routine examination of serum

specimens for evidence of treponemal disease

At the present time tests for treponemal antibody—TPI agglutination, complement fixation—are of greatest practical value as an aid in identifying patients with biologic false positive tests for Wassermann antibody Persons who show positive tests for Wassermann antibody in the absence of clinical or epidemiologic evidence suggesting syphilis should have their serum tested for the presence of treponemal antibody before a diagnosis of syphilis or other treponemal disease is established

Serologic tests have been used traditionally as a guide to therapy in syphilis and related diseases but their interpretation is fraught with difficulty It is a moot question whether antibody production continues for long after the antigenic stimulus provided by the treponeme has been eliminated Data from experimental animals with well-established infections indicate that following curative treatment Wassermann antibody declines fairly rapidly but treponemal antibody remains present for long periods although declining gradually in titer

There is some evidence suggesting that the same phenomena occur in human beings on an extended time scale but the data are not conclusive Following successful treatment of early syphilis both Wassermann antibody and treponemal antibody decline over a period of months the former more rapidly than the latter so that eventually most of such patients show negative serologic tests In more long-standing cases the decline in both types of antibody is much more gradual in many patients tests for Wassermann antibody eventually become negative whereas in most individuals tests for treponemal antibody remain positive It can be argued that the persistence of these antibodies reflects persistence of infection but clinical and immunologic evidence does not indicate that this is necessarily true There is much indirect evidence suggesting that, just as tetanus toxoid induces specific antibodies that persist for many years both Wassermann and treponemal antibody also may persist for many years after elimination of all treponemes by penicillin therapy

Therefore in the present state of our knowledge it would be unwise to assume that individuals who have received what is regarded as adequate treatment for syphilis and still show the presence of these antibodies are still infected Perhaps the best serologic guide to adequate therapy is evidence of some decline in titer of these various antibodies recognizing that such a decline may be detectable only over a period of years and not necessarily over a period of weeks or months

Spinal Fluid Tests Under normal circumstances the serum antibody does not pass into the spinal fluid even in syphilitic patients with a high serum titer In patients with central nervous system syphilis the antibody appears in the cerebrospinal fluid either because of damage to the blood brain barrier or through local elaboration Therefore the presence of a positive test for Wassermann or treponemal antibody in the spinal fluid is usually indicative of involvement of the central nervous system. The antibody titer of the fluid coupled with its cell content and protein content, and their response to antisyphilitic treatment provide information of diagnostic and prognostic value Tests for treponemal antibody have had less thorough clinical evaluation than have tests for Wassermann antibody

Treatment

Until the discovery and the development of penicillin the arsenicals notably arsphenamine and its derivatives in conjunction with bismuth were the drugs of choice in the treatment of syphilis Now these have been entirely superseded by penicillin which has been shown to be far more active as a treponemicidal agent as well as much less toxic for the patient.

Penicillin was first shown to be effective against *T pallidum* by Mahoney Arnold and Harris (1943) its use has revolutionized the therapy of syphilis and other treponemal diseases Instead of months and often years of therapy with arsenicals and heavy metals all of which were inherently toxic to the patient therapy is now accomplished in most patients within a period of 2 weeks although much longer periods of observation are desirable

Patients with early or latent syphilis are treated with 2 doses of a long lasting penicillin 2 4 million units in each dose given intramuscularly 2 weeks apart Benzathine penicillin G fortified with procaine is the preparation of choice, although procaine penicillin in oil containing 2 per cent of aluminum monostearate in the same dosage is satisfactory For children the dose of penicillin should be adjusted to 50 000 units per Kg of body weight

In patients with central nervous system syphilis or other forms of late syphilis it is customary to give a total of 4 doses of 2 4 million units each although evidence for the necessity of this increased amount is not altogether convincing (Hahn *et al* 1956 1959)

Tetracycline drugs have some treponemicidal action but are far less effective than penicillin These drugs should be used when penicillin is contraindicated because of hypersensitivity rather than the more toxic arsenicals and the less effective bismuth (Olansky and Garson 1958)

Epidemiology and Control

Syphilis is acquired principally by sexual contact extragenital infection accounts for less than 10 per cent of the total Primary and secondary syphilis in which there are open lesions discharging millions of organisms are the most infectious stages but patients may be infectious for months and occasionally for several years after most of the secondary lesions have disappeared spontaneously

Infection may be transmitted to the fetus in utero years after initial infection of the mother it is not known whether infection of the fetus occurs through transient spirochetemia in the mother or direct passage of treponemes through the uterine wall

The control of syphilis is made particularly difficult because many infected individuals are not aware that they have the disease this is particularly the case in females in whom the early lesions may be entirely within the genital tract and largely symptomless Infected prostitutes and other promiscuous women constitute especially important sources of infection because of the large number of individuals whom they may expose to infection

The widespread availability of penicillin has placed a potent weapon in the hands of physicians and public health officials in the control of syphilis Control methods which were applied intensively during and following World War II, led to a marked reduction of syphilis in most developed countries Concomitant with relaxation of intensive application of these control methods there has been clear indication of resurgence of syphilis as an endemic disease The next generation may well see more of this problem than the present one (Joint Statement, 1963) An important factor in control is the early and adequate treatment of the syphilitic patient in order to minimize the length of time during which he remains infectious thereby reducing the number of persons to whom he can transmit the disease Each case is potentially the source of a small outbreak and not infrequently 10, 20 or even more cases may be traced through several generations of infection to a single individual The sources of infection should be traced to bring under treatment the infected individual from whom the patient acquired his disease, who often is the focal point for many other actual and potential infections The contacts to whom the patient may have transmitted the disease should also be found and followed both clinically and serologically Many will have clinical evidence of the disease by the time they can be brought in for examination Obviously their early and adequate treatment will prevent them from transmitting the infection to others Those contacts who are clinically and serologically normal at the time of the first examination may develop the disease subsequently and should be kept under observation for several months In such persons treatment with a relatively small amount of penicillin may effectively abort an infection that is not evident at the time of the first examination

Methods of prophylaxis applicable principally to males have likewise been developed as a control measure Calomel ointment applied to the genitalia after sexual exposure has been used for many years Various other prophylactic measures include thorough washing of the genitalia with soap and water mechanical protection by use of the condom during exposure and the ad

ministration of penicillin by mouth or injection It is probable that each of these measures either alone or in combination with one or more of the others is effective in many instances but fails in others Since the infecting treponeme probably moves beyond the point of inoculation within 1 or 2 hours local prophylaxis must be applied promptly after exposure Perhaps the greatest limiting factor in the efficacy of prophylactic measures is failure of the individual to use them properly because of lack of knowledge—or simply neglect to use them at all The striking increase in the incidence of venereal disease in the Armed Forces immediately after the end of World Wars I and II affords adequate evidence of the practical limitations of prophylaxis

Although penicillin has been used successfully to prevent the development of infection in the sex contacts of proved cases of early syphilis its routine prophylactic use (e g in the Armed Forces as a substitute for local chemical prophylaxis) is not practicable The limiting factor is the slow rate at which *T pallidum* is killed by penicillin The single oral dose which suffices to prevent gonorrhea would be ineffective here and repeated dosages by mouth or parenteral administration would be difficult to employ as a prophylactic procedure

TREPONEMA PERTENUE AND YAWS
(Synonyms Frambesia Pian)

History

Yaws has been recognized as a clinical entity for centuries *Treponema pertenue* the causative organism was first identified in the lesions of yaws by Castellani soon after the discovery of *T pallidum* It is not known whether the disease was brought to the West Indies by infected African slaves or was indigenous to both parts of the world Yaws is largely confined to the tropics

Biologic Properties of T Pertenue

The organism is indistinguishable from *T pallidum* in many of its characteristics including morphology motility staining properties ability to induce Wassermann and treponemal antibodies in man and experimental animals and susceptibility to arsenical drugs and penicillin Like *T pallidum* *T pertenue* has not been cultivated on artificial media it can be maintained on Nelson s medium (see p 576) for several days and virulence is maintained for years when frozen at the temperature of dry ice (approximately −70 C) It does not survive freezing and desiccation

T pertenue has the same host range as *T pallidum* and the evolution of the experimental disease in the animal host follows much the same pattern as experimental syphilis However the character of the lesions tends to differ from those of syphilis particularly in that the yaws lesions in general are much less indurated the collections of round cells are more focal and there is strikingly less mucoid material in the lesions In rabbits for example intratesticular inoculation of yaws treponemes commonly gives rise to multiple small focal lesions in the visceral tunic of the testis the so called granular periorchitis which while it does occur occasionally after the inoculation of *T pallidum* is rare in experimental syphilis Similarly in hamsters the intracutaneous inoculation of *T pertenue* gives rise to a local lesion at the site of injection in the great majority of animals at the same time treponemes are demonstrable in the regional lymph nodes whereas following intradermal inoculation of *T pallidum* the organisms are regularly found in the regional lymph nodes but lesions at the site of injection are uncommon (Turner and Hollander 1957) The basis of these differences is not clear although it may be related in some as yet indefinable way to the observed difference in the amount of hyaluronic acid in the lesions of the two infections (see further discussion of this subject in the section Biologic Relationships Within the *Treponema* Group) Infection with *T pertenue* leads to the induction of both Wassermann and treponemal immobilizing antibodies in experimental animals and man Generalized lesions in rabbits are less common in yaws than in syphilitic infections The basis for these differences is obscure

The Disease in Man

In man the initial lesion or mother yaw appears 3 to 4 weeks after exposure as a painless yellow red papule (framboise or raspberry) surrounded by an inflammatory zone This gradually increases in size erodes

and ulcerates the dried exudate forming a dark crust From 6 weeks to 3 months later sometimes after the mother yaw has healed completely generalized secondary lesions develop which differ in no important respect from the primary lesion When they localize in mucocutaneous junctions (mouth nose perineum) the lesions are moist and resemble syphilitic condyloma Successive crops may appear over a period of several months to several years Plantar papules and hyperkeratosis of the soles of the feet—the so called crab yaws—are among the most common and the most incapacitating lesions

The late sequelae of yaws are generally restricted to skin and bone Gummatous nodules and deep chronic ulcerations or crippling bone and joint lesions may develop A destructive ulcerative mutilation of the rhinopharynx (gangosa) a proliferative exostosis of the upper maxilla (goundou) and juxta articular nodules are also ascribed to yaws Visceral complications are rare Although aortic and central nervous system involvement have been described most workers agree that such complications are rare and certainly much less frequent than in syphilitic infection Congenital yaws is believed not to occur

Immunity

In both animals and man one attack of yaws may confer protection against a second attack Immunity develops slowly thus reinoculation in man in the first 3 years of the infection may result in a modified attack but most infected persons are refractory to reinfection after 10 years There is considerable evidence that yaws confers a measure of protection against syphilitic infection In man almost all observers have commented on the relatively small number of cases of syphilis seen in population groups heavily infected with yaws Findlay and Wilcox (1945) produced a syphilitic infection by the subcutaneous inoculation of *T pallidum* into a subject with a clear history of yaws 10 years previously

Likewise there is evidence that human beings who have had syphilis have some degree of immunity to yaws Jalnel and Lange (1928) and Strong (1942) could not induce yaws lesions in persons with general paresis Turner (1936) found that of 10 individuals who presumably had latent syphilis all were refractory to inoculation with yaws treponemes, as were 9 of 10 other individuals who had yaws in the remote past while darkfield positive lesions were induced by the same inoculum in 9 of 10 patients who had had recent yaws infection

There is also a large body of evidence indicating the existence of a degree of cross immunity in the experimental infections When rabbits are infected with *T pallidum* and the disease is permitted to evolve to the point of latency a majority of the animals develop no lesion on challenge inoculation of yaws treponemes Likewise, yaws infected animals which have had the disease 3 months or longer commonly show some degree of immunity on challenge inoculation with *T pallidum* On the whole syphilis animals exhibit evidences of a higher degree of immunity to yaws than yaws animals to syphilis while the results vary according to the experimental methods employed taken as a whole published data provide incontrovertible evidence of a reciprocal cross immunity between yaws and syphilis For a review of pertinent experiments see Turner and Hollander 1957 Treponemal antibodies occurring during yaws infection in both man and animals have the capacity to immobilize syphilis treponemes and serum from syphilitic animals and man regularly immobilize yaws treponemes (Khan Nelson and Turner 1951)

Diagnosis

As in syphilis the diagnosis of yaws depends on (1) the appearance of the lesions (2) demonstration of treponemes in early lesions by darkfield examination and (3) serologic tests The florid skin lesions of yaws and the plantar lesions can scarcely be confused with any other disease Darkfield examination and serologic tests assist in differentiating yaws from nontreponemal disease but not of course from syphilis or other treponematoses

Epidemiology

Yaws is virtually limited to tropical areas and occurs principally where the rainfall is high In some areas fully 75 per cent of all

individuals have had yaws by age 20 Areas of high endemicity frequently lie within a few miles of communities in which yaws is un common The disease occurs principally among the lower socioeconomic groups of the population living under poor hygienic conditions but in areas of high endemicity well to do families may be affected

The disease is spread by direct contact with an open lesion that is discharging trepo nemes The organism apparently cannot pass through the intact epithelium and a cut or an abrasion perhaps only microscopic prob ably serves as a portal of entry The role of flies as a vector has been suggested by Kumm and Turner (1936) who found that a fly (*Hippelates pallipes*) widely distributed in the West Indies fed in large numbers on the open ulcerative lesions and that *T pertenue* could then be found in the foregut or the stomach where it remained viable for about 7 hours The regurgitation of infective ma terial on a breached area of the skin has been shown to cause infection in rabbits *T pertenue* has also been reported to pass through the intestinal canal of certain species of African flies and mosquitoes in viable form Although man can be infected at any age more than two thirds of the infections occur before the age of puberty and males are infected more commonly than females There is no convincing evidence of variation in susceptibility according to race

Treatment and Control

Yaws responds to the same therapeutic agents as syphilis There is some clinical evi dence to indicate that smaller amounts of these drugs are required in yaws than in syphilis but there are no carefully controlled clinical or experimental studies to support this view The trivalent arsenicals the heavy metals such as bismuth and mercury and various antibiotic drugs including penicillin tetracycline and oxytetracycline all have therapeutic action Penicillin is by far the most effective of all known therapeutic agents and is the drug of choice in treatment

Based on experience gained in many cam paigns the WHO Expert Committee on Venereal Infections and Treponematoses rec ommends a single injection of 1 2 million units of procaine penicillin in oil with 2 per cent aluminum monostearate (PAM) for adults with active yaws This dose is reduced proportionately for younger age groups for patients with latent yaws and for contacts of infectious cases In some campaigns lower doses have been used with apparent success Benzathine penicillin G a longer acting prep aration may prove to be even more effective

Because yaws occurs principally among peoples of rural tropical areas where there is limited access to medical facilities attempts at control usually have taken the form of a mass campaign too often these have been one shot campaigns based entirely on mass treatment without adequate preliminary sur vey or organized follow up As originally de veloped by the Jamaica Yaws Commission (Turner *et al* 1935) to be effective con trol campaigns should consist of (1) sur vey of a given area by sanitary inspectors for the purpose of detecting all infectious cases (2) treatment of these cases to render them noninfectious and (3) close observation of the community by paramedical personnel in order to discover and treat new infectious cases as soon as they occur An educational program pitched at an appropriate level should be conducted as an integral part of the campaign (Hackett and Guthe 1956)

TREPONEMA CARATEUM AND PINTA

(Synonyms Mal del pinto Carate)

Pinta is a disease characterized in its later stages by the presence of coalescing depig mented and mottled areas on the wrists the hands the ankles the feet and the scalp and hyperkeratoses of the palms and the soles It is prevalent in Mexico and Colum bia is encountered in most of the American tropics and recently has been reported from the Philippines Africa India and the islands of the South Pacific The number of cases in Central and South America is now esti mated at approximately a million Originally considered a fungus infection its treponemal origin was indicated in 1938 by Saenz Tri ana and Alfonso who demonstrated the or ganism in exudates from lesions and in fluid expressed from the adjacent lymph nodes

The organism is morphologically indistin guishable from *T pallidum* Already a con

and ulcerates, the dried exudate forming a dark crust From 6 weeks to 3 months later, sometimes after the mother yaw has healed completely generalized secondary lesions develop which differ in no important respect from the primary lesion When they localize in mucocutaneous junctions (mouth nose perineum) the lesions are moist and resemble syphilitic condyloma Successive crops may appear over a period of several months to several years Plantar papules and hyperkeratosis of the soles of the feet—the so called crab yaws—are among the most common and the most incapacitating lesions

The late sequelae of yaws are generally restricted to skin and bone Gummatous nodules and deep chronic ulcerations or crippling bone and joint lesions may develop A destructive ulcerative mutilation of the rhinopharynx (gangosa) a proliferative exostosis of the upper maxilla (goundou) and juxta articular nodules are also ascribed to yaws Visceral complications are rare Al though aortic and central nervous system involvement have been described most workers agree that such complications are rare and certainly much less frequent than in syphilitic infection Congenital yaws is believed not to occur

Immunity

In both animals and man one attack of yaws may confer protection against a second attack Immunity develops slowly thus re inoculation in man in the first 3 years of the infection may result in a modified attack but most infected persons are refractory to reinfection after 10 years There is consider able evidence that yaws confers a measure of protection against syphilitic infection In man almost all observers have commented on the relatively small number of cases of syphilis seen in population groups heavily in fected with yaws Findlay and Wilcox (1945) produced a syphilitic infection by the sub cutaneous inoculation of *T pallidum* into a subject with a clear history of yaws 10 years previously

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Epidemiology

Yaws is virtually limited to tropical areas and occurs principally where the rainfall is high In some areas fully 75 per cent of all

demic among nomadic tribes situ in British West Africa dichuchwa in Bechuanaland njovera in Southern Rhodesia and endemic syphilis in certain sections of the Balkans While the clinical and epidemiologic patterns of these syndromes differ somewhat, from the standpoint of causative treponemes sero logic tests and response to therapy they cannot be distinguished from one another or from yaws and syphilis

BIOLOGIC RELATIONSHIPS WITHIN THE TREPONEMA GROUP

On the basis of comparative studies in vitro and in vivo of 70 strains of treponemes derived from human beings with various treponemal syndromes in many parts of the world Turner and Hollander (1957) concluded that all strains were closely related in their essential biologic characteristics in the disease picture they induced in man and in experimental animals in their immunologic features and in their reaction to antibiotics However certain relatively stable differences were observed particularly in the lesions in rabbits the disease picture in hamsters and certain immunologic patterns as determined by challenge inoculation of rabbits

On the basis of these criteria strains of treponemes from various parts of the world were placed into one of three categories (1) syphilislike (2) yawslike and (3) an intermediate group which partook of some of the characteristics of both syphilis and yaws Most of the strains from the nonvenereal treponematoses including some of the yaws strains belonged to this intermediate group

These groupings were believed to reflect fundamental biologic differences in the strains of treponemes and it was postulated that the differences resided in the character and the amount of capsular mucopolysaccharide that each strain produced Six strains of *T cuniculi* did not clearly fit into any of the above three categories and may perhaps be regarded as a fourth group

THE BORRELIA

Introduction

The genus *Borrelia* comprises several species of spirochetes which are morphologically similar but exhibit widely different pathogenic proclivities and host range The most important members of the group from a medical standpoint are those causing relapsing fever in man closely related organisms cause a somewhat similar spirochetosis in fowls These organisms are primarily blood parasites Another large and perhaps heterogeneous subgroup are the borrelia associated with ulcerative conditions of the oral cavity and the genitalia they are also found in lung abscesses and in so-called tropical ulcers of the lower legs and the feet although here their etiologic role is not clear

The borrelia group of spirochetes are distinguished from treponema and leptospira by being longer as a rule with the spirals deeper more loosely wound and more flexible These organisms stain well with ordinary aniline dyes but are not readily cultivable on artificial media The type species of the human relapsing fever spirochetes is *Borrelia recurrentis* many other species that affect man have been identified and named but, as pointed out below it is questionable whether these should be regarded as separate species or as variants of the one type species *B gallinarum* is the type species of the fowl spirochetoses and *B vincenti* of the spirochetes that occur in debilitated tissue lesions of human beings

BORRELIA RECURRENTIS AND RELAPSING FEVER

History

In the 18th century relapsing fever was often confused with typhus fever but the two were clearly distinguished by Henderson in 1843 The spiral organism causing the disease *B recurrentis* (obermeieri) was first seen in the peripheral blood of a human patient by Obermeier in 1868 and the disease was reproduced in man by the injection of infected blood in 1874 In 1904 Ross and Milne showed that so-called African tick fever was also caused by a spiral organism demonstrable in the blood and they indicated its probable identity with relapsing fever Shortly thereafter it was shown that as had been suspected the body louse could also act as a vector (Moursund 1942)

Morphology

B recurrentis is a highly flexible spiral or

fusing multiplicity of names has been applied to it among them *T carateum T herrejoni T pictor T pintae* and *T americanum* Al though it has not yet been cultivated on arti ficial media its regular presence in the le sions and the successful transfer of the dis ease from man to man (Leon Blanco 1939) with the recovery of the same organism from the induced lesions seems to establish its causal relationship A rabbit is said to have been inoculated successfully with or ganisms from a human lesion although 4 rabbits subinoculated from that animal did not develop a lesion a human subject simul taneously inoculated did develop a typical darkfield positive primary lesion in 47 days (Leon Blanco and Otéiza 1945)

Numerous other attempts to infect rabbits and hamsters have failed although in a few instances motile treponemes have been re covered from the regional lymph nodes of hamsters several weeks after inoculation In fected human beings develop both Wasser mann antibody and treponemal antibody that immobilize *T pallidum* (Varela and Olarte 1950) Although *T carateum* un doubtedly belongs to the group of treponemal organisms the limits of its biologic relation ship to *T pallidum* and *T pertenue* are still vague

The disease may be contracted at any age and is first evidenced by a nonulcerating pri mary lesion This is followed in 5 to 18 months by the appearance of successive crops of flat erythematous and hyperpig mented lesions (pintid) After several years one observes the characteristic late depig mentation and hyperkeratoses Although late visceral complications have been re ported definitive evidence is lacking

Human beings have been successfully in oculated with the exudates from the lesions but reinfection does not succeed in the late cases Syphilitic subjects have been success fully inoculated with pinta and subjects with pinta may contract syphilis (Gonzalez Her rejon 1940) indicating at least some im munologic differentiation between the two infections

Transmission is not venereal but usually occurs by person to person contact Flies (*Hippelates*) allowed to feed on serous fluid containing the treponemes have been shown to be capable of transmitting the disease to man (Leon Blanco and Soberon Parra 1941)

The disease responds to treatment with arsenicals, bismuth and penicillin as do yaws and syphilis Penicillin is the drug of choice administered in the same dose and in the same manner as for syphilis

TREPONEMA CUNICULI AND RABBIT SYPHILIS

Treponema cuniculi was identified by Bayon in 1913 as the cause of a natural in fection of rabbits The organism is morpho logically indistinguishable from *T pallidum* which it resembles also in its susceptibility to arsenicals and penicillin. The natural lesion consists of superficial scaly, eroded lesions on the genitalia and the adjacent perineal region Inoculation into the skin of that area reproduces the disease and intratesticular inoculation causes in 14 to 28 days an in flammatory reaction resembling that caused by syphilis but less extensive lacking the induration characteristic of the latter and consisting largely of fine nodules in the parietal layer of the tunica vaginalis without marked enlargement of the testis (McLeod and Turner 1946) In the following 1 to 6 months metastatic lesions may be seen in the testis the scrotum the anus the prepuce and the glans and secondary lesions in the skin and the mucocutaneous borders of the eyes the nose and the mouth Wassermann antibody develops in the same proportion and to the same degree as in rabbits infected with *T pallidum* and *T pertenue* Trepone mal antibody to *T pallidum* also develops but apparently in lower titer than to *T cuniculi* (Khan *et al* 1951)

OTHER TREPONEMATOSES OF MAN

In many geographic areas of the world particularly where the majority of people are economically depressed and live under unhygienic conditions endemic treponemal infections are observed Transmission is commonly nonvenereal occurring through direct bodily contact or through the medium of common eating and drinking utensils These nonvenereal treponematoses have often been given local names such as bejel in Syria and adjacent areas where it is en

toneally Guinea pigs are susceptible to the tick borne strains the organisms surviving in the brain for more than 3 years (Sergent 1945) while louse borne strains are only rarely infectious on direct inoculation into this species (Coghill and Gambles 1948) Rabbits cats and dogs are not susceptible Monkeys show the characteristic relapsing course of the human disease Organisms appear in the blood in 24 to 48 hours and disappear spontaneously in 2 to 5 days In young rats (40 to 80 Gm) they may appear in the blood in huge numbers within 24 to 72 hours and disappear with extraordinary rapidity within a few hours Even in these animals however the disease is rarely fatal Consistent with the demonstration of the organisms in the cerebrospinal fluid and the brain in human cases there is some evidence that in rats mice and guinea pigs the brain may serve as a reservoir of infection after the apparent disappearance of organisms from the circulating blood (Heronimus 1928 Anderson 1946 Schuhardt and Hemphill 1946)

IMMUNITY

Serum agglutinins and bactericidal antibodies are readily demonstrable in both the experimental and the human disease and the termination of the individual febrile attacks as well as the eventual cure may be related to their appearance It is significant that the organisms which appear during a febrile relapse often differ in their serologic reactivity from those present in the immediately preceding attack (Cunningham Theodore and Fraser 1934) In rats a protein deficient diet has been found to cause a significant increase in the severity of infection and mortality (Guggenheim Buechler-Czaczkes and Halevy 1951) In squirrels inoculated with a single human strain Meleney (1928) isolated 6 serologically distinct strains during a series of relapses There is no fixed order in which the mutant strains appear in the course of a single infection but there is an indication that the organisms in alternate relapses may be more closely related in their antigenic structure than is the intervening mutant Therefore the development of mutant strains not susceptible to the antibody previously elaborated may be the actual cause of the relapse and final cure may reflect an increasingly broad immunity afforded by the multiple mutant strains The antigenic variation in successive relapses has been observed even after single-cell inoculation (Schuhardt and Wilkerson 1951) However it must be noted that Ashbel (1942) has observed relapses without demonstrable changes in immunologic reactivity and Stein (1944) has furnished evidence of a common antigen in a number of supposedly different strains isolated from both animals and man

Recovered patients have been found to resist reinfection from 2 to 5 years later However other workers have found that patients remained immune only so long as the organisms persisted in the tissues Attempts to immunize animals with killed spirochetes have been unsuccessful and it has not yet been possible to characterize and differentiate strains on the basis of their antigenic reactivity There is evidence of cross protection between tick borne and louse borne strains Monkeys infected with the tick borne California strain proved to be resistant to reinfection with a louse borne Chinese strain and in hamsters when the order of inoculation was reversed the second infection consisted of a single short attack, with no relapses (Chen Zia and Anderson 1945)

DIAGNOSIS

The diagnosis rests primarily on the demonstration of the organism in the blood by direct darkfield observation, by the examination of stained blood films or by animal inoculation Young white rats weighing from 30 to 80 Gm are particularly susceptible organisms appearing in the blood in large numbers in 24 to 72 hours When one centrifuges the blood of infected animals or man the organisms tend to concentrate in the white blood cell layer facilitating their detection by darkfield examination or stain In louse borne infections Proteus OXK agglutinins in high titer appear in a substantial proportion of the cases (Zarofonetis *et al* 1946 Davis 1948)

TREATMENT

The trivalent arsenical drugs particularly neoarsphenamine were used for many years but now have been superseded by penicillin

ganism, varying in length from 8 to 30 microns and in thickness from 0 2 to 0 5 micron with 5 to 10 irregular and loosely wound spirals which average 1 to 2 microns in depth and 3 microns in width The or ganisms are actively motile, with both ro tational and transitional movement How- ever the latter is not always progressive, and in blood preparations the organisms may move back and forth within the same micro scopic field Unlike *T pallidum B recur rentis* stains well with the usual bacterial stains as well as with Wright s Giemsa and other stains containing the aniline dyes The electron microscopic studies of Swain (1955) show a central axial filament with a sur rounding sheath of protoplasm On wash ing in distilled water this sheath disappears and numerous fibrils resembling flagellae are seen These fibrils may arise from the axial filament or be the remains of the sheathlike envelopes Division is believed to be by trans verse fission

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Despite reports from time to time of cul tivation of virulent relapsing fever organisms on artificial media cultivation is not regu larly successful The organisms do grow well in the chick embryo (Oag 1940) and direct isolation from the blood of patients has been accomplished by this method (Bohls *et al* 1940 Chen 1941) Inocula tion can be made onto the chorio allantoic membrane or into the body of the embryo When planted on media containing blood serum or ascitic fluid and maintained at re frigerator temperature relapsing fever spiro chetes may remain motile and infective for many months (Wolman and Wolman 1945) Their virulence is maintained for long periods at the temperature of dry ice (Turner and Fleming 1939)

The widespread distribution of the dis ease and the several modes of transmission have led to the isolation of a large number of strains differentiated primarily on the basis of the area of isolation or the vector concerned in their transmission rather than by inherent biologic differences in the or- ganisms Although some strains apparently will grow preferentially in certain species of ticks, this may not reflect a genetic difference Louse borne strains have been shown to be infective for ticks, which then can transmit the disease to man and naturally tick borne strains have been similarly transferred to lice but there is a question concerning the epidemiologic importance of the unnatural vector

Although agglutinins and other antibodies can be produced in high titer the serologic differentiation of the various strains is com plicated by the fact that their antigenic struc ture apparently changes repeatedly during a single infection

Pathogenesis

The louse and tick borne infections can not be distinguished with certainty on the basis of their clinical manifestations The dis ease in man usually begins with an acute febrile onset from 3 to 10 days after infec tion In this initial febrile stage there are usually large numbers of organisms in the blood they may be found in the urine in approximately a fourth of the cases and by animal inoculation sometimes spirochetes can be demonstrated in the cerebrospinal fluid After an average of 4 days the fever declines coincident with the disappearance of organisms from the blood As the number of organisms decreases, they become less motile tend to assume bizarre forms and may agglutinate in rosettes During the afebrile period the blood is not infectious for lice The afebrile period may last from 3 to 10 days and is followed by a second febrile attack during which organisms reap pear in the blood but in smaller numbers There may be from 3 to 10 such recurring febrile attacks The mortality in the endemic infection varies between 2 and 5 per cent but in epidemics it may be 50 per cent or even higher In fatal cases organisms are found in sections of the spleen and the liver and are particularly numerous in the malpighian bod ies of the spleen which show miliary necrotic lesions Hemorrhagic lesions may be found in the gastrointestinal tract and in the kidney There have been occasional reports of relaps ing fever in the offspring of infected mothers presumably caused by transplacental inocula tion

Monkeys mice and rats can be inoculated subcutaneously intravenously or intraperi

toneally Guinea pigs are susceptible to the tick borne strains the organisms surviving in the brain for more than 3 years (Sergent 1945) while louse borne strains are only rarely infectious on direct inoculation into this species (Coghill and Gambles 1948) Rabbits cats and dogs are not susceptible Monkeys show the characteristic relapsing course of the human disease Organisms appear in the blood in 24 to 48 hours and disappear spontaneously in 2 to 5 days In young rats (40 to 80 Gm.) they may appear in the blood in huge numbers within 24 to 72 hours and disappear with extraordinary rapidity within a few hours Even in these animals however the disease is rarely fatal Consistent with the demonstration of the organisms in the cerebrospinal fluid and the brain in human cases there is some evidence that in rats mice and guinea pigs the brain may serve as a reservoir of infection after the apparent disappearance of organisms from the circulating blood (Heronimus 1928 Anderson 1946 Schuhardt and Hemphill 1946)

Immunity

Serum agglutinins and bactericidal antibodies are readily demonstrable in both the experimental and the human disease and the termination of the individual febrile attacks as well as the eventual cure may be related to their appearance It is significant that the organisms which appear during a febrile relapse often differ in their serologic reactivity from those present in the immediately preceding attack (Cunningham Theodore and Fraser 1934) In rats a protein-deficient diet has been found to cause a significant increase in the severity of infection and mortality (Guggenheim Buechler-Czaczkes and Halevy 1951) In squirrels inoculated with a single human strain Meleney (1928) isolated 6 serologically distinct strains during a series of relapses There is no fixed order in which the mutant strains appear in the course of a single infection but there is an indication that the organisms in alternate relapses may be more closely related in their antigenic structure than is the intervening mutant Therefore the development of mutant strains not susceptible to the antibody previously elaborated may be the actual cause of the relapse and final cure may reflect an increasingly broad immunity afforded by the multiple mutant strains The antigenic variation in successive relapses has been observed even after single-cell inoculation (Schuhardt and Wilkerson 1951) However it must be noted that Ashbel (1942) has observed relapses without demonstrable changes in immunologic reactivity and Stein (1944) has furnished evidence of a common antigen in a number of supposedly different strains isolated from both animals and man

Recovered patients have been found to resist reinfection from 2 to 5 years later However other workers have found that patients remained immune only so long as the organisms persisted in the tissues Attempts to immunize animals with killed spirochetes have been unsuccessful and it has not yet been possible to characterize and differentiate strains on the basis of their antigenic reactivity There is evidence of cross protection between tick borne and louse borne strains Monkeys infected with the tick borne California strain proved to be resistant to reinfection with a louse borne Chinese strain and in hamsters when the order of inoculation was reversed the second infection consisted of a single short attack with no relapses (Chen Zia and Anderson 1945)

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TABLE 1 GEOGRAPHIC DISTRIBUTION OF VARIOUS SPECIES OF *Borrelia* RESPONSIBLE FOR RELAPSING FEVER AND THEIR VECTORS

	Species of *Borrelia**	Mode of Transmission: Louse borne (*Pediculus humanus*)	Mode of Transmission: Tick borne (*Ornithodoros*)
Europe	*recurrentis*	+	
	obermeieri	+	
	hispanica		*O erraticus (marocanus)*
			O verrucosus
Africa	*duttoni (crociduri)*		*O moubata*
	kochi		*O erraticus (marocanus)*
	russi		
	berbera	+	
	aegyptica		
	turicatae		*O turicata*
	marocana		
	sogdiana		*O savignyi*
Middle East	*persica*		*O papillipes (tholozani)*
			O asperus
			O lahorensis
India	*carteri*	+	*O thola ani*
			O crossi
			O lahorensis
Russia	*latyshevi*		*O verrucosus*
			O neerensis
			O tartakovskyi
			O thola ani
North America	*novyi*		
	turicatae		*O turicata*
	parkeri		*O parkeri*
	hermsii		*O hermsi*
Central and South America	*dugesii*		*O dugesi*
	venezuelense		*O venezuelensis (rudis)*
	neotropicalis		*O talaje*
	turicatae		*O turicata*

Validity of identification as separate species questionable

Montana Idaho Texas Kansas California Colorado Arizona Utah Florida New Mexico and Oklahoma) Ground squirrel and prairie dog burrows are sometimes heavily infested with ticks with a high incidence of spirochetes suggesting that these and other rodents may serve as natural reservoirs of infection

Although the causative organisms cannot pass through the intact skin they can penetrate the mucous membrane (e g conjunctiva) and transplacental infection may also occur The possible role of the bedbug in transmission has been suggested No effective vaccine is available and chemoprophylactic measures are not practicable In louse borne epidemics the isolation of patients large scale treatment and the mass delousing of the population as with DDT are effective procedures However DDT resistant strains of lice have developed in many areas and this form of control is now less dependable

In endemic areas reduction of the tick population by periodic spraying of living quarters with benzene hexachloride a chemical long used in tick control of cattle has been reported to effect a major reduction in the morbidity of the disease (Holmes 1953)

and the tetracyclines However, few well controlled clinical or experimental studies have been made with these drugs

Penicillin appears to be effective when given in large doses—1,000 000 units of crystalline penicillin per day in aqueous solution in divided doses every 3 hours for at least 5 days Failures have been attributed to inadequate dosage However the treatment of choice is chlortetracycline in doses of 0 5 Gm every 6 hours for 5 days then 1 0 Gm twice daily for another 5 days Children under 10 should be given half this dose Oxytetracycline in approximately the same dosage as chlortetracycline seems to be equally effective on the basis of limited trials Streptomycin also seems to have some therapeutic value Exacerbation of symptoms (Herxheimerlike reaction) frequently occurs immediately following the initiation of drug therapy (Cherry 1955)

Epidemiology and Prevention

Human borrelia infections have been reported from all parts of the world except Australia New Zealand and Oceania Relapsing fever is transmitted from man to man from animal to animal and from animal to man entirely by insect vectors While many arthropod insects are capable of natural infection transmission to man is primarily by the body louse and by a variety of ticks and the epidemic pattern is determined largely by the prevalence of these vectors and opportunities for man to be parasitized by them Thus great epidemics of louse borne relapsing fever have occurred one of the most recent being the great epidemic wave that swept over North Africa in 1942-44 in which an estimated 1 million persons became ill of whom 50 000 died (Gaud and Morgan 1947) Louse borne relapsing fever may occur concomitantly with louse borne typhus fever leading to considerable diagnostic confusion Tick borne relapsing fever is largely endemic in occurrence, although its prevalence in some areas notably central Africa has reached almost epidemic proportions at times

In northern and western Africa in Europe and in parts of Asia the disease is spread primarily by the body louse *Pediculus humanus* which becomes infectious 4 to 5 days after the ingestion of infected blood and remains infectious for 2 to 3 weeks The infection in lice is not transmitted to the second generation (Wolman and Wolman 1945) The disease apparently is transmitted when lice are crushed near a bite or scratch which provides a portal of entry for the organism It is the louse borne infection which may become epidemic under conditions which lower the host resistance and favor the rapid multiplication and the wide dissemination of the insect vector Thus epidemics generally have occurred in malnourished overcrowded populations with poor personal hygiene and often have been incidental to famine and war The seasonal incidence, with increased spread in cold weather, probably reflects the heavier louse infestation and the crowding together, for warmth of thickly clothed persons

In the endemic areas of Central and South Africa over a wide area of Asia and in the Americas the disease is tick borne The disease is by no means uncommon in the United States In Texas alone 100 cases were diagnosed during the period June 1942 to May 1949 and it seems likely that many additional cases escaped detection The most important vector is the genus *Ornithodoros* many species of which have been shown to be infected in nature (Table 1) There appears to be a remarkable species specificity in this host parasite relationship There is some evidence that rodents may serve as natural reservoirs of infection for the tick Organisms are found in all parts of the infected tick may persist for years and are transmitted to the ova for many generations their infectivity for mice remaining unchanged However the percentage of transovarian transmission varies widely in different tick species (Davis 1939 1948) Some tick species appear to transmit infection to man through the coxal fluid while others introduce the organisms directly by a bite The tick borne disease is not usually epidemic and the seasonal incidence probably is related to the prevalence of the vector Infected ticks (*O turicata O hermsi O parkeri* and *O talaje*) have been found over a wide area of the United States (Wyoming

found to be leptospiral rather than viral infections (Beeson and Hankey 1952 Gauld *et al* 1952) A recent addition to this list and one of the most unexpected is Fort Bragg fever or pretibial fever first encountered at Fort Bragg North Carolina (Bowdoin 1942 Daniels and Grennan 1943) It was then thought to be a self limiting virus infection but following several years of serial passage through guinea pigs and hamsters it was discovered by Gochenour and his associates (1952) that the infectious agent so propagated was not a virus but a strain of leptospira not previously encountered in the United States This strain differs serologically from *L icterohaemorrhagiae* and *L canicola* but resembles *L autumnalis* originally isolated in the Far East

The continuing identification of leptospira as the etiologic agent in a number of obscure infections coupled with the protean and often minimal clinical aspects of the disease indicate that human leptospirosis already known to be world wide in distribution is probably far more common than has hitherto been suspected and that the possibility of its presence should be duly considered in infections of obscure etiology At least 7 antigenically distinct strains are known to exist in North America All have been isolated from cases of human infection as well as from the animal species which seems to be its more usual host See also the recent review of Alston (1961)

Morphology and Cultivation

All the pathogenic leptospira are indistinguishable morphologically and in most other biologic characteristics except antigenic structure The description which follows will refer specifically to the type species *L icterohaemorrhagiae* but applies to other species unless otherwise noted

L icterohaemorrhagiae is characterized by its extraordinarily fine spirals so closely wound and so short that they may be visible on darkfield examination only as a series of small dots and usually are not distinguished in stained films The length of the organism varies from 4 to 20 microns It is approximately 0 1 to 0 2 micron in width and moves by the active rotation of one end of the organism bent into a hook Both ends may be motile in which case the actively rotating organism has no translational motion Considerable confusion and mistaken diagnoses in the past originated in the strands which under in vitro conditions frequently extend from the surface of red blood cells and break off to float free in the plasma While not actually spiral in form they are extremely difficult to distinguish under the dark field microscope from leptospira even in expert hands therefore identification of leptospira in human blood specimens is unreliable and probably never should serve as the basis for diagnosis of the disease

Electron microscope studies (Czekalowski and Eaves 1955 Simpson and White 1961) show a central cylindrical core containing focal areas of increased density which may be nuclei This core is wound in helical fashion around a much smaller but more rigid axis There is also an external sheath of poor electron density bound by a thin external membrane Knoblike structures often occur at the ends of the axis but it is not known whether these have any function in the reproductive process it is believed that division is usually by transverse fission No structural differences have been noted among various serotypes

Leptospira are aerobic and grow best at 28 to 30 C Various media have been described most of which consist of serum saline enriched with peptone or meat infusion base in 0 2 per cent agar Chang (1947) recommends the addition of a small amount of an emulsion of fresh guinea pig liver to maintain virulence Leptospira will also grow in simple media consisting of salts thiamine asparagine and rabbit serum albumin (Schneiderman *et al* 1953 Johnson and Gary 1962 Vogel and Hutner 1961) More recently isolated colonies have been obtained on semisolid media (Cox and Larson 1957 Armstrong and Goldberg 1960) Growth is also obtained on the chorio allantoic membrane of the chick embryo (Morrow *et al* 1938 Chabaud 1939)

SPECIES OF LEPTOSPIRA

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BORRELIA VINCENTI AND ULCERATIVE LESIONS OF THE OROPHARYNX THE GENITALIA AND THE EXTREMITIES

In a diverse group of infections (tropical ulcer Vincent s angina or ulcerative stomatitis pulmonary spirochetosis ulcerative lesions of the genitalia), one finds large numbers of a delicate short (5 to 10 microns), actively motile spiral organism with a variable number of shallow irregular turns It is usually present in association with a coarse, thick gram negative rod with tapered ends which is often banded or beaded in stained preparations (*Bacillus fusiformis*) There is no agreement as to whether these two organisms are the actual cause of the necrotic lesions or only secondary invaders Thus the infection has been ascribed by some to a herpeslike virus (Black, 1942) and by others to a predisposing vitamin or other dietary deficiency which would permit the invasion of the tissues by normally harmless organisms of the surface of the mucous membranes It should be noted that these same organisms can be recovered from about the gingival margins of almost all healthy adult human beings sometimes in large numbers in which case there is usually an associated pyorrhea (Thomson 1956 Ngu 1960)

These organisms appear to respond satisfactorily to the broad spectrum antibiotics particularly chloramphenicol and oxytetracycline (Ampofo and Findlay 1951) Skin grafting may be necessary (Nelson and Semambo, 1956)

LEPTOSPIRA AND THE LEPTOSPIROSES

Introduction

The genus *Leptospira* comprises a group of spiral organisms which in morphology, biologic characteristics and antigenic structure have little in common with either the *Treponema* or the *Borrelia* group of spirochetes Unlike the borrelia and the pathogenic treponema they can be cultivated readily on artificial media A number of species of *Leptospira* have been identified each with fairly distinctive characteristics in respect of antigenic structure and often their host range but all the leptospira cause an acute illness in man which, while it may vary in severity, is similar in its general pattern regardless of the species involved It appears, too that wild rodents are the natural hosts and the essential reservoirs of all the pathogenic leptospiral organisms Spirochetes with the morphology of leptospira may rarely be found about the gingival margins and in other chronic ulcerative lesions and leptospira (*L biflexa*) have been cultured from tap water but little is known about the antigenic structure of these presumably non pathogenic varieties

The historical association of jaundice with leptospiral infection stemming from the classic description of the disease by Weil and designation of the first pathogenic species isolated as *L icterohaemorrhagiae* probably has contributed to the tardy recognition in the United States at least, of the true prevalence of leptospiral infections Jaundice while a prominent clinical sign in many severe cases, is present in less than half of all recognized cases of leptospirosis

History

Spirochetal fever (also termed spirochetal jaundice or Weil s disease) was first described in 1886 as a febrile disease associated with jaundice and characterized by involvement of the kidney and the spleen The causative organism was isolated in 1915 by Inada and his co workers who also demonstrated the role of rats as natural vectors inoculated guinea pigs from infected rats and demonstrated the organism in each stage of that cycle

It has since developed that there are many different leptospiral species, which vary in their geographic distribution host range and antigenic structure Many and perhaps all of these are pathogenic for man and over the past 2 decades a number of previously obscure infections have been identified as leptospiral in origin The marsh or field fever of central Europe (*L grippotyphosa*) the 7-day fever of Japan (*L hebdomadis*) and swineherd s disease first seen in Australia and since recognized in both Europe and the United States (*L pomona*) are cases in point A significant proportion of cases of so-called aseptic meningitis have also been

found to be leptospiral rather than viral infections (Beeson and Hankey 1952 Gauld *et al* 1952) A recent addition to this list, and one of the most unexpected is Fort Bragg fever or pretibial fever first encountered at Fort Bragg North Carolina (Bowdoin 1942 Daniels and Grennan 1943) It was then thought to be a self limiting virus infection but following several years of serial passage through guinea pigs and hamsters it was discovered by Gochenour and his associates (1952) that the infectious agent so propagated was not a virus but a strain of leptospira not previously encountered in the United States This strain differs serologically from *L icterohaemorrhagiae* and *L canicola* but resembles *L autumnalis* originally isolated in the Far East

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Table 2 Serologic Classification of Some of the Leptospiral Species Pathogenic for Man*

Serogroups	Immunologically Related Species	Isolated Year	Isolated Country	Recovered From	Known Distribution
†*L icterohaemorrhagiae*		1914	Japan	Rats mice other rodents swine foxes apes dogs cats opossum horses poultry calves muskrat	World wide
†*L canicola*		1931	Holland	Dogs golden hamsters	World wide
	L salinem	1923	Dutch East Indies	Rats guinea pigs	Southeast Asia
	L ballum	1943	Denmark	Mice white rats rabbit guinea pigs opossum raccoon skunk fox wildcat	North America, Denmark France
L hebdomadis		1918	Japan	Field mice dogs opossum raccoon skunk	Japan Eastern Asia Indonesia Europe North America
	L sejroe	1937	Denmark	Field mice	Central Europe Indonesia
	L saxkoebing	1942	Denmark	White rats field mice	Central Europe
L pomona		1937	Australia (Queensland)	Swine cattle dogs raccoon skunk wildcat	Australia Indonesia Middle East Central Europe North and South America
	L bovis	1948	USA	Cattle goats sheep	USA Europe Israel
L grippotyphosa		1928	Russia	Field mice other rodents raccoon skunk	South and East Europe Africa Israel Southeast Asia USA
	L bovis (palestinense)	1947	Palestine	Cattle goats sheep field mice chickens	USA Europe Israel
	L geffeni	1950	Israel	Goats cattle voles mice	Israel
L autumnalis		1925	Japan	Field mice rats dogs guinea pigs	Japan Southeast Asia Indonesia USA Switzerland
	Fort Bragg Strain	1952 (1943)	USA	Opossum raccoon	USA
L australis		1937	Australia (Queensland)	Field rats shrew dogs swine guinea pigs opossum raccoon	Australia Southeast Asia Japan Central Europe
	L pyrogenes	1922	Indonesia	Field rats guinea pigs bandicoot swine	Australia Southeast Asia Italy North America
L mitis		1940	Australia (Queensland)	Cattle swine	Switzerland Italy Australia
	L hyos	1944	Argentine	Swine cattle horses opossum raccoon skunk wildcat	Argentine France North America
L batavia		1926	Netherland East Indies	Rats cats field mice guinea pigs swine dogs	Indonesia Malaya Europe Central Africa

* Compiled largely from Schlossberger and Brandis 1954

† These two subgroups are closely related serologically

certain related historical and epidemiologic data (Walch Sordrager 1939 Wolff 1953 Schlossberger and Brandis 1954) but the classification given is far from definitive Wolff (1953) for instance gives somewhat different relationships in a more complicated scheme As our knowledge increases doubtless both the geographic and the natural host ranges of these species will be found to be more extensive All the pathogenic species seem to be distinct from the nonpathogenic *L biflexa* found the world over in small streams lakes and stagnant water but even these two groups have certain antigens in common The differentiation of the pathogenic strains is complicated by the marked degree of cross reactivity indicative of a considerable overlapping in antigenic structure and necessitating quantitative serologic tests and in particular careful antibody absorption studies Fractionation studies indicate that a somatic antigen probably a lipopolysaccharide is genus specific while a surface antigen is species specific The latter as yet chemically undefined may be recovered from culture supernates as well as from the organisms themselves (Rothstein 1957)

Therefore the division of this group of organisms into species and subspecies is made on a rather arbitrary basis and the studies of Bessemans and his associates (1947) and Pike and Schulze (1958) in which exposure to specific antisera led to antigenic variants suggest that similar antigenic instability might occur in nature About 60 serotypes have been distinguished

Antibody studies have been carried out principally with agglutination lysis and complement fixation technics using whole cultured organisms as antigens Soluble antigens have also been used in complement fixation and precipitin tests The agglutination lysis test using living organisms as antigen is the test that is used most frequently although organisms killed by heat or formalin have also been employed It is unfortunate that more definitive studies on killed antigens or antigenic fractions have not been made since the lack of readily available living cultures of various species of leptospira has greatly limited the use of tests for leptospiral antibody in clinical and epidemiologic practice

Other technics have been proposed but their uses and limitations have not been clearly defined Among these are the complement fixation test employing sonically disrupted leptospiral antigens (Randall Wetmore and Warner 1949) hemagglutination of human O or sheep erythrocytes following sensitization with extracts of leptospira (Chang and McComb 1954 Cox 1955) and the use of infrared spectrophotometry for the differentiation of leptospiral species (Schneider and McLaughlin 1955) Alexander and his associates (1956) have recently demonstrated a soluble thermolabile oxygen stable hemolysin in certain species which is highly toxic for experimental animals (Sleight and Langham 1962)

Host Range and Pathogenesis

It is probable that wild rodents constitute the natural reservoir of all leptospiral species although some species have not yet been recovered from these animals In the United States several studies have shown that from 40 to 60 per cent of wild rats are naturally infected with leptospira (Li and Davis 1952) In most of these studies the infecting organism has been considered to be *L icterohaemorrhagiae* but only limited studies have been made to ascertain whether this is the only species of leptospira commonly carried by rats As indicated in Table 2 several species of leptospira have been recovered from mice field rats and other small rodents Many species of wild and domesticated animals also have been found to be naturally infected but like man these are probably aberrant hosts rather than important reservoirs of infection (Alston 1961 Gorman *et al* 1962 Evans *et al* 1962)

Of the common experimental animals the guinea pig and the Syrian hamster are the most susceptible the young of each species being much more susceptible than the adult animal Some species of leptospira notably *L icterohaemorrhagiae* appear to produce a more severe disease in guinea pigs and hamsters than others but it is not certain that this constitutes a reliable differential point for some strains of all species induce a mild and largely symptomless infection in these animals while an occasional strain of many species induces a more severe infection

Packchanian (1940) found 26 species and subspecies of American rodents to be susceptible to *L icterohaemorrhagiae* infection death occurring in 3 to 13 days usually preceded by jaundice Following intraperitoneal intramuscular or subcutaneous inoculation of infective material young (3 to 6 weeks old) guinea pigs and hamsters develop fever and loss of weight within 3 to 5 days, followed by jaundice and multiple hemorrhages in the skin the subcutaneous tissue and the muscles Leptospira can be demonstrated readily in the blood the peritoneal fluid or the homogenates of the liver or the spleen In mild infections recovery is rapid, and leptospira can be recovered only early in the infection

Wild rats and other small rodents cannot be used experimentally because of the likelihood of natural infection and even laboratory stocks have been found to be infected Infection is rarely fatal in these animals and it is probable that they remain infected for life There is frequently an extensive and long lasting kidney involvement which can be demonstrated readily by darkfield examination culture or guinea pig inoculation and the animal may excrete leptospira in the urine during the rest of its life Water and food contaminated by the urine of infected rodents appear to be the principal means of spread to other animals and man

It is not known whether the initial site of multiplication of leptospira in infected animals and man is the blood or one or more of the internal organs but early in the disease leptospira can be recovered from the blood often the urine and from the liver the spleen the muscles and the serous surfaces including the meninges and the cerebrospinal fluid The liver and the spleen enlarge and an obstructive type of jaundice develops Petechial hemorrhages in the muscles, the skin the sclerae etc are among the most common manifestations of the disease in both man and animals (Stavitsky 1945)

Leptospirosis in Man

Various clinical syndromes have become associated in the literature with a particular species of leptospira but as time goes on these syndromes appear less and less to be specific to any one species (Edwards and Domm, 1960) a possible exception may be pretibial fever Regardless of the species of leptospira involved, the disease in man is characterized by an acute febrile illness muscle pains and headache, albuminuria and the occurrence of multiple small hemorrhages which may be particularly noticeable in the sclerae In the classic Weil s disease originally believed to be due only to *L icterohaemorrhagiae* jaundice was a prominent sign, but it is now known that many individuals infected with this species of leptospira do not have jaundice while some patients infected with the other types do present this sign In general perhaps *L icterhoaemorrhagiae* and *L canicola* tend to give rise to a more severe type of human disease than do the other species although undoubtedly many mild cases of all types go undiagnosed evidenced by the occurrence of high titer agglutinins in individuals who have had no illness especially suggestive of leptospirosis (Ward and Turner 1942, Miller 1961) There is probably some degree of meningeal involvement in most cases and a not insignificant proportion of cases of aseptic meningitis have been found to be due to leptospirosis

Even in cases which can reasonably be attributed to a single species the disease in man has all degrees of severity varying from infections so mild as scarcely to attract the attention of the patient and recognizable only by serologic test to serious sometimes fatal illnesses with deep jaundice and profound prostration It usually begins after an incubation period of 6 to 15 days as an acute febrile illness which runs an irregular course some patients relapsing after an afebrile interval Conjunctivitis (episcleral injection) is a prominent and almost pathognomonic symptom The central nervous system may be involved with the appearance of the organism in the cerebrospinal fluid Meningeal involvement is particularly common in infections with *L icterohaemorrhagiae L grippotyphosa L canicola and L pomona* and several outbreaks of leptospiral meningitis have been described (Gauld *et al* 1952 Beeson and Hankey 1952) It seems clear that not only the severity of the disease but also its manifestations may vary widely in different areas and in different outbreaks and that the care used in looking for the

disease affects the number of relatively mild cases discovered The febrile illness subsides by lysis after 3 to 10 days and may be followed by a second bout of fever In fatal cases there are hemorrhagic lesions in the kidney the liver the skin the muscles or the central nervous system The mortality is ex tremely variable ranging from 4 to 50 per cent in different outbreaks with an average of 5 to 10 per cent

Diagnosis

Definitive diagnosis rests on demonstration of leptospira or the appearance of specific antibodies in the serum Early in the disease the organisms may be demonstrated in the blood by animal inoculation or by growth in culture the use of both methods concur rently yields a greater number of positive results than either method alone Young guinea pigs or hamsters are inoculated intra peritoneally with 0 5 ml of citrated whole blood or blood plasma after light centrifu gation the weight of the animal is taken daily and when the animal ceases to gain or loses weight it is sacrificed and peritoneal fluid blood or suspensions of liver are ex amined under the darkfield for leptospira Subculture of the test animal s blood will also yield a few positive isolations that otherwise would be missed Direct examination of the patient s blood is unreliable because of the difficulty of differentiating leptospira from red cell strands (Wolff 1954 Babudieri 1961)

The proportion of patients with demon strable leptospira in the blood declines sharply during the second and the third weeks of ill ness but at this time the organisms may be recovered from the urine It is necessary to alkalinize the urine since leptospira rapidly disintegrate in a acid medium

Antibodies to the specific type of leptospira appear from the 7th to the 14th day of disease and rise sharply as demonstrated by the agglutination lysis or the complement fixation test the former test is regarded as the more reliable A significant rise in anti body is particularly convincing Cross reactions between species of leptospira occur but it is desirable to use antigens prepared from several species In the United States antigens prepared from *L icterohaemorrhagiae* *L pomona* and *L autumnalis* should be used for ordinary diagnostic studies there is usually considerable serologic crossing between *L icterohaemmorrhagiae* and *L canicola*

Immunity

The decrease in the number of organisms after the first 7 to 10 days probably re flects the development of specific antibodies The serum of convalescent patients protects guinea pigs and mice against an otherwise fatal inoculum and will cause lysis of the leptospira both in vitro and in vivo Ag glutinating antibodies have been found to persist in the blood of recovered patients for many years Hyperimmune rabbit anti serum may have agglutination titers of 1 10 000 000 and 0 3 ml of such serum administered even 72 hours after inoculation may cure the infection in white mice

Artificial immunization has been carried out in animals and in human beings in a few areas Reactions in man tend to be severe and on the whole active immuniza tion is not recommended However Wani (1933) claimed reduction in the incidence of Weil s disease following vaccination of coal miners in Japan with killed *L ictero haemorrhagiae* and numerous workers have demonstrated satisfactory rises in leptospiral antibodies following vaccination with heat killed organisms (Borg Peteresen 1953 Babudieri *et al* 1955 Parnas *et al* 1959) Animals both experimental and domestic have been immunized with heat killed and formalin killed organisms with what appear to be successful results Alston and Broom 1958 Alston 1961)

Treatment

No specific treatment yields dramatically effective results (Kocen 1962) Specific anti serum therapy and the use of the sulfona mides have been discarded While favorable results with one or another antibiotic have been reported the results of carefully con trolled experiments have been disappointing Penicillin is the drug of choice

Penicillin in amounts as small as 0 4 units per ml (about 0 25 mcg per ml) will inhibit the growth of *L icterohaemorrhagia* in culture (Alston and Broom 1958) but the

drug is not leptospiricidal even in large amounts The tetracyclines also inhibit growth in vitro in rather small amounts (0 2 to 20 mcg per ml) but leptospiricidal levels are far higher than those clinically attainable in man (500 to 700 mcg per ml)

In a controlled series of 79 patients treated with various antibiotics reported by Smadel (1953) no clear-cut response to the drugs could be demonstrated In a more recent series Kocen (1962) treated 28 patients with penicillin and compared the results with those in 33 patients treated in the same hospital without antibiotics Penicillin significantly re duced the duration of pyrexia and may have otherwise beneficially affected the course of the disease In this series soluble penicillin 600 000 units was given intramuscularly at 4 hour intervals for 24 hours then at 6 hour intervals for 4 days for a total of 13 2 million units

Epidemiology and Preventive Measures

Leptospirosis is primarily a zoonosis and man is an aberrant host Wild rodents appear to be the principal reservoirs it is not clear whether certain domestic animals known to be frequently infected with leptospira should be regarded as primary or secondary reser voirs The organisms are communicated to man either directly or indirectly by way of water or soil although leptospira do not sur vive long in nature outside an animal host The organism presumably enters the body through small breaks in the skin or through intact mucous membranes particularly the conjunctiva (Stavitsky 1945)

Many more animal species can be infected experimentally than have been found infected in nature For example leptospira have been transmitted experimentally to ticks (*O moubata*) which th n became capable of infect ing guinea pigs although neither of these species has been found infected in nature

In general, leptospiral infection in rodents is symptomless but experience in the labora tory with hamsters suggests that the young of the species may be more susceptible

Leptospiral infection of domestic animals has become associated with certain sero types but the more that infection is recog nized the more are exceptions to the general rule discovered Thus infections in dogs usually have been with *L canicola* or *L icterohaemorrhagiae* but most other species have been recovered occasionally (Alexander *et al*, 1957) cattle and horses also have been found infected with almost all species of leptospira

The similarity of the clinical syndrom s in man caused by different species of leptospira has already been referred to and it is probable that epidemiologic syndromes also are no more specific but merely reflect opportunities for infection The commonest epidemiologic pattern presents a situation in which man comes into frequent contact with water contaminated by leptospira-containing urine of infected rodents or domestic ani mals (Wolff and Ruys, 1953) The oppor tunities for this occurrence are countless and the wonder is that human beings ar not infected more often Thus the risk is comparatively great among sewer and mine workers soldiers in trench warfare bathers in swimming holes and canals workers in cane and rice fields and poultry dressers For example it has been shown that the sudden outbreak of human cases in the rice fields of northern Italy corresponds to the invasion of these fields by mice early in July During this period great numbers of young mice are born so that the surface water is contaminated with urine containing lepto spira (Mino 1942) Likewise Kathe (1951) demonstrated that epidemics of swamp fever occurred in Silesia only when (1) abundant rainfall was coincident with an epizootic in field mice (2) agricultural areas were cov ered by flood water and (3) the mean daily temperature was not lower than 18 to 19 C On the other hand domestic animals may cause leptospirosis in man independent of conditions of the climate and the soil Infec tion may occur through contact with infected animals urine or by handling the flesh of diseased animals Bernkopf and his associ ates (1947) have shown that refrigerated meat of bovine carriers may remain infective for 33 hours after slaughtering The inci dence of leptospirosis in man may change in any region according to changing conditions of animal husbandry

Leptospirosis can occur at all seasons in all age groups and in both sexes however for the reasons given above it is a disease primarily of warm weather, with highest in

cidence among young male adults and certain occupational groups Preventive measures vary according to (1) the ecology of the carrier host (2) the mode of transmission of infection and (3) the population endangered and they must be developed to meet the different epidemiologic patterns encountered Elimination of rat infestation in eating establishments and dwellings will prevent a mall proportion of cases Infections from bathing in public pools can be prevented by denying access to rats through proper sanitation and screening (Walch Sorgdrager 1939) Little can be done to reduce the risk of bathing in natural streams and stagnant pools except to warn individuals of the risk (Gauld *et al* 1952) The risk of infection among sewer workers and swineherds is greatest for those who go bare foot wearing strong boots is therefore a preventive measure Leptospirosis has been controlled among poultry dressers and fish mongers by correction of unsanitary conditions in which offal rats and standing water provided opportunities for the spread of infection (Davidson and Smith 1939 Ward *et al* 1942) A marked reduction in the incidence of canefield fever has been obtained by burning the dry leaves of standing cane which grows in low lying areas Burning is done just before cutting and does not damage the canes it drives out rats and evaporates rat urine and small pools of surface water (Broom 1953) Protection of the hands by heavy gloves has been recommended for butchers and other workers who handle potentially infected meat

Of the three links in the chain of transmission—the carrier the leptospira outside the body of the carrier and the susceptible host—preventive measures have been directed most often against the center link by ratproofing or by the wearing of protective clothing Vaccines have probably not reached a stage of practical efficacy although promising results have been reported in man and in animal (Alston and Broom 1958 Alston 1961)

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days Their habitat is the intestinal tract of man and animals where for the most part they have no significance as causative agents of gastrointestinal disease The paracolon bacilli will be discussed as to their relationship to other enteric bacilli *Klebsiella pneumoniae* is encapsulated and nonmotile and a saprophyte of the intestinal and the upper respiratory tracts but may cause inflammatory lesions in the lower respiratory tract and elsewhere *Donovania granulomatis* will be described as a relative of the *Klebsiella* These coliform organisms and those of the *Proteus* and the *Pseudomonas* groups have assumed increasing importance as causative agents of disease since the introduction of antibiotics as therapeutic agents particularly in persons already affected by other diseases (Finland *et al* 1959) This seems to have been due to alterations in the bacterial flora of the host with predominance of these enteric bacilli particularly those which are naturally resistant to commonly used antibiotics or readily become so due to the selection of resistant mutants when the antibiotics are employed in therapy

The *Salmonella* include the causative agents of typhoid fever paratyphoid and other enteric fevers gastroenteritis various types of septicemic infections and certain diseases of the lower animals as well Characteristically they fail to ferment lactose and *Salmonella typhosa* and a few other salmonella organisms including *Salmonella gallinarum* do not produce gas in the fermentation of any sugars

The *Shigella* include the causative agents of bacillary dysentery in man In contrast with those of other groups these organisms are nonmotile and with one important exception fail to ferment lactose and are separated into two groups on the basis of the fermentation of mannitol All dysentery bacilli fail to produce gas from fermented sugars

The *Proteus* group is comprised of organisms widely distributed in nature Although they are frequently found in the intestinal tract of man their causal relationship to enteric disease is with one exception doubtful Elsewhere in the body they may cause primary or secondary infections especially in the genitourinary tract They are actively motile decompose urea and fail to ferment lactose

For the sake of convenience *Pseudomonas aeruginosa* and *Alkaligenes fecalis* will be considered at the end of this chapter

The biochemical reactions of the enteric bacteria are presented in Table 1

THE COLIFORM BACILLI

The coliform group includes the following gram negative bacilli *Escherichia coli* *Aerobacter aerogenes* the paracolon bacilli and *Klebsiella pneumoniae* The first two are often referred to as the colon aerogenes group *E coli* is a normal inhabitant of the intestinal tract of man and animals *A aerogenes* is found most frequently on grains and plants but also occurs in the feces of man and animals Because of its predominantly intestinal origin *E coli* is used as an indication of pollution of water with fecal material whereas the presence in water of other bacteria which like *A aerogenes* have other natural habitats does not necessarily indicate fecal pollution

Organisms of the colon aerogenes group cause infections in man which are primarily of a localized nature often involving the genitourinary tract or organs having an anatomic relationship to the intestinal tract e g gallbladder peritoneum and appendix

Escherichia Coli

E coli (synonym *Bacterium coli*) was isolated from feces by Escherich in 1885 It is found universally in the intestinal tract of man and animals and being the predominant organism in the colon is commonly referred to as the colon bacillus

E coli is a gram negative bacillus which commonly occurs as short rods from 2 to 3 microns long and about 0 6 micron in breadth and may form chains Occasionally very long filamentous forms are seen Most strains are motile *E coli* does not produce spores Some strains possess a definite capsule and growth at low temperatures favors the demonstration of this structure (Morgan and Beckwith 1939) which may be identified by specific serologic reactions (e g capsular swelling)

E coli is facultatively anaerobic and

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25

The Enteric Bacteria

INTRODUCTION

The enteric group of bacteria (*Enterobacteriaceae*) includes a large number of species of gram negative nonsporulating rods whose natural habitat in most instances is the gastrointestinal tract of man and other animals However this designation fails to acknowledge that other groups of organisms such as bacteroides enterococci and clostridia also are found in the gut and actually outnumber the so-called enteric bacteria Some of the enteric bacteria are pathogenic for man and cause various types of gastrointestinal diseases such as typhoid and other enteric fevers gastroenteritis (*Salmonella*) or dysentery (*Shigella*) Others (e g the *Escherichia*) appear to lead a saprophytic existence in the intestinal tract but may cause pathologic processes in other parts of the body such as the genitourinary and the respiratory systems In nature the *Aerobacter* group occurs most commonly in soil and on grain though often it is isolated from the gut There are no simple differential criteria for these enteric organisms and classification is based on morphologic characteristics biochemical reactions antigenic properties and ecologic considerations Even when all these criteria are invoked some organisms fail to exhibit all the characteristics of a single group appearing to occupy an intermediate position between the main groupings The discovery that characteristics of one enteric bacillus can be transferred to related organisms by transduction with bacteriophage and recombination probably accounts for the presence of these intermediate organisms Because of these intermediate organisms it is difficult to classify enteric bacilli into definitive taxonomic groups However, the organisms can be separated into general groups which have practical usefulness in the understanding of these organisms and their relationship to disease

As a general rule the enteric bacilli grow readily on ordinary media They are aerobes or facultative anaerobes and characteristically ferment a wide range of carbohydrates Many are actively motile and at least one group commonly possesses easily demonstrable capsules Their antigenic structure forms a complex mosaic which often results in serologic interrelationships between different genera and species

The enteric bacteria will be considered under the following groups

The coliform group is characterized by the prompt fermentation of lactose usually with the production of acid and gas *Escherichia coli* is a common inhabitant of the intestinal canal but frequently causes infections of the urinary tract and other organs The closely related organism *Aerobacter aerogenes* is found most frequently in soil and on grain Certain other closely related bacilli are usually classified with these organisms The paracolon bacilli ferment lactose slowly frequently only after incubation for several

days Their habitat is the intestinal tract of man and animals where for the most part they have no significance as causative agents of gastrointestinal disease The paracolon bacilli will be discussed as to their relation ship to other enteric bacilli *Klebsiella pneumoniae* is encapsulated and nonmotile and a saprophyte of the intestinal and the upper respiratory tracts but may cause inflammatory lesions in the lower respiratory tract and elsewhere *Donovania granulomatis* will be described as a relative of the *Klebsiella* These coliform organisms and those of the *Proteus* and the *Pseudomonas* groups have assumed increasing importance as causative agents of disease since the introduction of antibiotics as therapeutic agents particularly in persons already affected by other diseases (Finland *et al* 1959) This seems to have been due to alterations in the bacterial flora of the host with predominance of these enteric bacilli particularly those which are naturally resistant to commonly used antibiotics or readily become so due to the selection of resistant mutants when the antibiotics are employed in therapy

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E coli is facultatively anaerobic and

grows on all ordinary laboratory media The optimum incubation temperature is about 37° C On beef extract agar it usually forms circular convex smooth colorless colonies with regular edges but some colonies, which probably represent rough dissociants, may have an irregular surface and edge By transmitted light the growth has a granular appearance On blood agar some strains produce hemolysis Growth in broth produces uniform turbidity with some sediment, the cultures have a characteristic fetid odor

E coli ferments a variety of carbohydrates including dextrose lactose, maltose, mannitol and xylose but not dextrin or starch with the production of acid and gas Sucrose salicin and raffinose are attacked by some strains but not by others Colon bacilli form indol and do not liquefy gelatin H S is not produced

Colon bacilli are killed at a temperature of 60° C for 30 minutes They are more susceptible than the salmonella to the inhibitory action of such compounds as brilliant green dye and sodium desoxycholate The composition of certain differential media which are designed for the isolation of salmonella from the feces is based on these differences in susceptibility

E coli is serologically heterogeneous and the antigenic pattern of the various species has been studied by Kauffmann (1951) who has established a diagnostic antigenic schema analogous to that available for the salmonella group he has divided them into O groups which are numbered 1 to 139 Besides the traditional O and H antigens capsular or envelope (K) antigens are recognized these are further subdivided into thermolabile (L and B) and thermostable (A) antigens The former are chiefly envelope antigens corresponding to the Vi antigens of salmonella In contrast the A antigens are usually of the visible capsular type Strains containing K antigens are more toxic and more resistant to the normal defense mechanisms of the host than are other strains Strains of *E coli* are classified on the basis of their O antigens and clinical evidence suggests that some of these O groups are more likely to be found in appendicitis peritonitis and infections of the genitourinary tract than are other strains suggesting a correlation between pathogenicity and antigenic structure Some strains of *E coli* have somatic antigens which are serologically identical with those occurring in certain members of the salmonella group

E coli undergoes dissociation to give rough and smooth colonial types The smooth round translucent colonies of the S form contrast with the irregular colonies of the R form which have a dull surface and opaque character Mucoid forms occur and appear more frequently when cultures are grown at low temperatures

Whereas *E coli* is generally a harmless and perhaps useful inhabitant of the intestines of man and animals under certain conditions it may assume the role of a pathogen especially in the invasion of organs anatomically related to the intestinal tract such as the appendix gallbladder, peritoneal cavity kidneys and bladder In appendicitis and peritonitis the colon bacillus commonly occurs in the tissues along with a variety of other organisms It is one of the most common invaders of the peritoneum following perforation of some part of the intestinal tract *E coli* appears to be the predominant organism in many cases of the suppurative form of cholecystitis Acute infections of the urinary tract including pyelitis pyelonephritis and cystitis are caused most frequently by *E coli* Along with the other organisms that may occur on the skin *E coli* is also found in wound infections but much less frequently than streptococci or staphylococci except when the wound has been contaminated with urine or feces Colon bacilli may gain access to the bloodstream particularly in infants in the agonal stages of diseases and immediately after death Furthermore with the use of antibiotics such as penicillin which have their most pronounced action on gram positive organisms gram negative organisms such as *E coli* may become predominant in the upper respiratory tract and cause pneumonia

Recent investigations have demonstrated that certain strains of *E coli* cause infantile diarrhea and gastroenteritis Bray (1945) Giles and Sangster (1948) and Taylor Powell and Wright (1949) have independently isolated a serologically distinct strain of the colon bacillus (0111) from the feces of

Table 1 Biochemical Reactions of Enteric Bacteria

Organism	Motility	Glucose	Lactose	Sucrose	Mannitol	Salicin	Rhamnose	Dulcitol	Inositol	Sorbitol	Arabinose	Xylose	Indol	Hydrogen Sulfide	Gelatin Liquefaction	Russell's Double Sugar: Slant	Russell's Double Sugar: Butt	Citrate Utilization	Acetyl Methyl Carbinol—(VP)	Methyl Red	Tri Methyl Amine Oxide Reduction
E coli	+	AG	AG	V	AG	V	AG	V	—	AG	AG	AG	+	—	—	A	AG	—	—	+	
A aerogenes	V	AG	AG	AG	AG	AG	AG	V	AG	AG	AG	AG	—	—	—	A	AG	+	+	—	
A cloacae	+	AG	AG	AG	AG	AG	AG	V		AG	AG	AG	V	—	+	A	AG	+	+	—	
Paracolon Group																					
Bethesda Ballerup	+	AG	(V)	V	+	V	+	V			+	+	—	+	—			+	—	+	
Arizona	+	AG	(V)	—	AG	—		—	—				—	+	+			+	—	+	
Providence	+	AG	—	(AG)	—	—		—	—				+	—	—			+	—	+	
Kl pneumoniae	—	AG	AG	AG	AG	AG	AG	V	V	AG	AG	AG	—	—	—	A	AG	+	—	—	
S typhosa	+	A	—	—	A	—	—	—	—	A	—	V	—	+	—	Alk.	A		—	+	
S paratyphi	+	AG	—	—	AG	—	AG	AG	—	AG	AG	—	—	—	—	Alk	AG		—	+	
S schottmuelleri	+	AG	—	—	AG	—	AG	AG	V	AG	AG	AG	—	+	—	Alk	AG		—	+	
S hirschfeldii	+	AG	—	—	AG	—		AG	—	AG	AG	AG	—	+	—	Alk	AG		—	+	
S typhimurium	+	AG	—	—	AG	—		AG	V	AG	AG	V	—	+	—	Alk.	AG		—	+	
S choleraesuis	+	AG	—	—	AG	—	AG	V	—	AG	—	AG	—	V	—	Alk	AG		—	+	
S enteritidis	+	AG	—	—	AG	—		AG	—	AG	AG	AG	—	+	—	Alk.	AG		—	+	
S gallinarum	—	A	—	—	A	—		A		—	A	A	—	V	—	Alk	A				
Sh dysenteriae Type 1	—	A	—†	—	—	—	—	—		—	—	—	—	—	—	Alk	A	—	—		—
Sh dysenteriae Type 2	—	A	—	—	—	—	V	—		V	V	—	+	—	—	Alk	A	—	—		—
Sh dysenteriae Types 3 7	—	A	—	—	—	—	V	(V)		V	A	V	V	—	—	Alk	A	—	—		—
Sh flexneri	—	A	—	V	A*	—	V	V		V	V	—	V	—	—	Alk	A	—	—		—
Sh boydii	—	A	—	—	A	—	—	V		(V)	A	(V)	V	—	—	Alk	A	—	—		—
Sh sonnei	—	A	(A)	(A)	A	—	A	—	—	—	A	V	—	—	—	Alk	A	—	—		+
Proteus vulgaris	+	AG	—	AG	—	AG	—	—	—	—	—	AG	V	+	+	Alk	AG	—	—	+	
Proteus morgani	+	AG	—	V	—	—	—	—	—	—	—	—	+	—	—	Alk.	AG	—	—	+	
Ps aeruginosa	+	A											—	—	+			+	—	—	
Alcaligenes faecalis	+	—	—	—	—	—	—	—	—	—	—		—	—	—	Alk	—				

A = Acid AG = Acid and gas Alk = Alkaline + = Positive — = Negative V = Variable ( ) = Delayed
* = A few strains fail to ferment mannitol
† = A few strains are late lactose fermenters

drates with production of acid and gas They are closely related to the aerobacter group They are found in the nose the mouth and the intestinal tract of normal persons in the lungs of patients with pneumonia and other respiratory diseases and in suppurative infections in other parts of the body

Klebsiella pneumoniae is the most important member of the group It was discovered in 1883 by Friedlander in the lungs of patients dying with pneumonia and is now known to cause a small proportion of the bacterial pneumonias

Morphology

In infected tissues *Kl pneumoniae* usually occurs as an ovoid rod from 2 to 5 μ long and 0 5 μ thick often in pairs In cultures it shows pleomorphism with curved rods long filaments and other forms It occurs naturally in the mucoid phase with a capsule which is usually visible even in an ordinary Gram stain and is particularly striking when the organisms are grown on media rich in carbohydrate A profuse mucoid growth of a tenacious character is produced on solid media It is luxuriant and viscous in broth After repeated subculture *Kl pneumoniae* tends to lose its mucoid character and dissociates to give smooth colonies made up of organisms which do not produce the characteristic large capsules Reversion to the mucoid form may occur on further subculture

Biologic Characteristics

Kl pneumoniae is facultatively anaerobic and grows best at 37° C with a range from 15° to 40° C Growth is luxuriant on ordinary nutrient media The organisms are killed by moist heat at 55° C in 30 minutes

The biochemical reactions are variable from strain to strain These variations may occur within a single serologic type and increase the difficulty of classification *Kl pneumoniae* ferments glucose maltose lactose sucrose mannitol and salicin with the production of acid some strains fail to produce gas Indol is not produced The organism is usually MR$^-$, VP$^+$ and does not produce H S

The morphologic and the biochemical properties of *Kl pneumoniae* show that it is closely related to *E coli* from which it is differentiated chiefly by its respiratory habitat, its etiologic role in certain cases of primary pneumonia in man and its characteristic easily visible capsule As noted previously it cannot be distinguished from *A aerogenes* and becomes indistinguishable from other coliform organisms when it undergoes dissociation from the mucoid to the smooth form and thereby loses its large capsule (Ostermann and Rettger 1941)

The antigenic structure of *Kl pneumoniae* has been studied by Julianelle (1926) who found that the capsule contains a type specific polysaccharide He described 3 serologic types, A B and C and a fourth heterogeneous group X The specific polysaccharide of the type B organism shows an immunologic relationship to that of the type 2 pneumococcus A nucleoprotein which is common to all types is found in the body of the bacterial cell

In more recent studies Kauffmann (1949) has identified somatic (O) antigens in *Kl pneumoniae* which permit the classification of this organism into 3 principal antigenic O groups These O antigens also occur in *A aerogenes* indicating again the close relationship between *Klebsiella* and *Aerobacter* species In addition Kauffmann has extended the studies of the capsular K antigens to describe a total of 14 types The same capsular antigen may be found in different O groups

Pathogenicity

Kl pneumoniae is found in the respiratory tract of about 5 per cent of normal individuals (Bloomfield 1921) and frequently occurs as a secondary invader in the lungs of patients with bronchiectasis influenza and tuberculosis It is the primary cause of pneumonia in less than 3 per cent of all bacterial pneumonias (Hyde and Hyde 1943) It has been isolated from patients with pleurisy appendicitis cystitis and pyelonephritis and from the feces of about 5 per cent of normal individuals (Baehr *et al* 1937) It occurs frequently in abdominal infections though its presence is often overlooked in the bacteriologic studies

In animals *Kl pneumoniae* has been isolated from spontaneous respiratory diseases

of mice and in a metritis occurring in mares Types A and B are highly pathogenic for mice by intraperitoneal injection while guinea pigs and rabbits show a higher degree of resistance Type C appears to be relatively avirulent for animals

In pneumonic infections in man *Kl pneumoniae* is notable for its destructive action on the tissues producing abscesses and cavities Julianelle (1941) found Type A in 64 per cent Type B in 14 per cent Type C in 7 per cent and Group X types in 15 per cent of a series of cases of pneumonia The mortality of untreated cases is high and may reach 90 per cent in those with bacteremia Chronic Friedlander bacillus infections of the lung may follow the acute pneumonia with production of cavities and thus require surgical intervention

Sulfadiazine and streptomycin have proved to be of value in therapy as well as chloramphenicol chlortetracycline and oxytetracycline Two or more drugs are usually employed for maximal therapeutic effectiveness

Two other encapsulated mucoid gram negative bacilli resembling *Kl pneumoniae* and occurring characteristically in the mucoid phase have been isolated from disease conditions of the upper respiratory tract of man Frisch in 1882 isolated an encapsulated organism from the granulomatous nasal lesions of patients with rhinoscleroma and a similar organism was isolated by Loewenberg in 1894 from the nasal secretions of individuals with ozena a fetid catarrhal condition of the nose The capsular antigen of the rhinoscleroma organism is similar to that of Type C Friedlander s bacillus but *Kl ozenae* may be easily differentiated from *Kl pneumoniae* and *Kl rhinoscleromatis* by serologic tests

DONOVANIA GRANULOMATIS AND GRANULOMA INGUINALE

From a venereal disease associated with chronic ulcerative lesions of the genital region in which smears of the lesions reveal short bacilli (Donovan bodies) within mononuclear cells Anderson *et al* (1945) isolated in chick embryos a short encapsulated gram negative rod *Donovania granulomatis* which was later cultivated on artificial media and found to resemble Friedlander s bacillus with which it has antigenic relationships On subsequent study no evidence has been obtained that this disease is transmitted primarily by sexual contact and since it occurs only in individuals with a low standard of cleanliness it is more prevalent in areas of poor economic status

THE PROTEUS GROUP

The proteus group consists of pleomorphic gram negative bacilli which do not ferment lactose and are characterized by their active motility and spreading growth on solid media They are commonly found in soil water sewage and manure and occur in normal human stools They are not usually pathogenic for man though they cause infections of the genitourinary and the gastrointestinal tracts The two commonest pathogenic species are *Proteus vulgaris* and *Proteus morgani* other strains are of medical importance because of their antigenic relationships to certain rickettsiae

Proteus Vulgaris

Proteus vulgaris is a motile gram negative rod shaped organism with great variation in size and shape The more typical forms in agar cultures average from 1 to 3 microns long and from 0 4 to 0 6 micron wide but short coccobacillary forms are also seen The rods occur singly in pairs and frequently in long chains Young cultures which show swarming are particularly pleomorphic and may include long filamentous forms

The organism is a facultative anaerobe with an optimum growth range from 34 to 37° C but able to grow well at 20 C on solid moist media It spreads rapidly from the initial colonies over the entire surface by a process called swarming which is due to its very active motility Swarming can be prevented by increasing the agar content of the media to 6 per cent In broth the organism gives a moderate uniform turbidity with some deposit

P vulgaris produces acid and gas in glucose sucrose and galactose Some strains ferment maltose The maltose fermenting strains which form indol are VP negative and usually fail to grow on citrate agar, while

drates with production of acid and gas They are closely related to the aerobacter group They are found in the nose the mouth and the intestinal tract of normal persons in the lungs of patients with pneumonia and other respiratory diseases and in suppurative infections in other parts of the body

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soil Some species are pathogenic and the type species *Pseudomonas aeruginosa* (*Pseudomonas pyocyanea Bacillus pyocyaneus*) occurs in human feces and in wound and urinary tract infections in man

Gessard in 1882 isolated *Ps aeruginosa* from the blue pus found in some wound infections This organism is closely related to about 30 other species of *Pseudomonas* which occur principally in soil water and sewage although some produce disease in animals and plants *Ps fluorescens* is one of the most common of these other species

Ps aeruginosa is a gram negative motile rod measuring from 1 5 to 3 0 microns by about 0 5 micron It is not encapsulated and forms no spores It grows readily on all ordinary culture media and has a sweetish odor On agar it forms round smooth moist glistening colonies which have a fluorescent yellowish green color although most of the pigment diffuses into the medium coloring it bluish green The organism is aerobic and grows best at 30° to 37 C It is killed at 55° C for 1 hour

It is not an active fermenter of carbohydrates and produces acid but no ga in glucose It actively liquefies gelatin produces ammonia and grows on citr ite medium *Ps aeruginosa* does not produce indol is methyl red and Voges Proskauer negative it fails to produce hydrogen sulfide and to reduce nitrates

The bluish green color produced by *Ps aeruginosa* consists of two substances pyocyanin a bluish green pigment soluble in chloroform and water and fluorescin which is greenish yellow fluorescent and soluble in water but not in chloroform The closely related organism *Ps fluorescens* forms only fluorescin These pigments are antibacterial for certain other organisms

Ps aeruginosa is the only member of this group which is pathogenic for man It is found occasionally in the human intestine and on the skin as well as in water and sewage It may produce local suppurative lesions especially skin and wound infections and otitis media. It may occur in infections of the genitourinary tract, the respiratory tract the joints and the eye Meningitis due to it has been observed to follow a lumbar puncture or operative exposure of the meninges In some outbreaks of a dysenterylike enteric infection *Ps aeruginosa* has been isolated under circumstances which suggest an etiologic role

With the more frequent use of antibiotics that eliminate many of the pathogens in respiratory and urinary tract infections *Ps aeruginosa* has assumed more importance etiologically since it emerges as the dominant organism when the more susceptible species disappear *Ps aeruginosa* may cause pneumonia in debilitated individuals receiving prophylactic penicillin or may appear as the causative agent in chronic pyelonephritis when other organisms have been eliminated by proper therapy It may also become the predominant organism on the injured skin of a burned patient receiving antibiotic therapy and give a blue green color to the surface exudate

It produces fatal infections when injected subcutaneously or intravenously into guinea pigs or rabbits

Streptomycin chlortetracycline and chloramphenicol have proved to be of some value in treating infections by *Ps aeruginosa* but polymyxin B and colistin generally exert greater therapeutic activity against this organism

Alcaligenes Faecalis

Alcaligenes faecalis is a gram negative rod which is found in human feces and can be confused with *Salmonella typhosa* since it does not ferment lactose and therefore produces similar colonies on the usual differential media used for the isolation of enteric pathogens It is readily distinguished from other gram negative organisms by its failure to ferment glucose as well as most other carbohydrates In rare instances it may cause enteric infections in man and is not uncommon as a cause of inflammation in the urinary tract

THE SALMONELLA SALMONELLOSIS AND TYPHOID FEVER

The salmonella are gram negative non spore forming, motile bacilli which are easily cultivated on ordinary media they characteristically fail to ferment lactose and sucrose The different species are closely

those which do not ferment maltose are indol negative, usually VP positive and usually grow on citrate agar *P vulgaris* exhibits active proteolytic action and liquefies gelatin, digests casein and decomposes urea It produces H_2S and NH_3 and reduces nitrates

The antigenic structure of *P vulgaris* has received considerable attention because of the use of certain strains designated by the letter X in the Weil Felix reaction for the diagnosis of typhus fever and of other rickettsial diseases The group is antigenically heterogeneous with differences in both H and O antigens On the basis of their serologic relationships to certain rickettsia the O antigens of the X strains have been divided into 3 types OX2 OX19 and OXK Castaneda (1934) reported the isolation from Proteus OX19 of a soluble specific polysaccharide which seems to be present also in *Rickettsia prowazecki* and to be responsible for the serologic reaction utilized in the Weil Felix test in epidemic typhus fever

Organisms of the proteus group are widely distributed in nature They are found in water and soil and form an important part of the flora of decomposing animal and vegetable matter in manure and sewage They occur in the feces of man and animals but in large numbers only when some abnormal condition exists They become more prominent when stool specimens are incubated in the enrichment media tetrathionate broth or selenite F used for suppressing *E coli* in the isolation of enteric pathogens In addition to its saprophytic existence *P vulgaris* may be isolated in pure or mixed cultures in urinary tract infections from abscesses or wounds and in peritonitis It causes up to 13 per cent of human urinary tract infections (Pierson and Honke 1941) and often appears in patients who have been treated successfully with various antibacterial agents for infections caused by other genitourinary pathogens It appears in large numbers on infected wounds after antibiotic therapy by reason of its ability to develop drug resistance and thus to overgrow the drug sensitive species *P vulgaris* has been isolated from cases of gastroenteritis where it appeared to play an etiologic role (Cooper *et al* 1941) Isolation of the X strains of proteus from the urine and the feces of patients with typhus fever led to the study of the possible (now discounted) etiologic relationship to typhus and to the discovery and the use of the Weil Felix reaction

The intraperitoneal inoculation of *P vulgaris* into mice, rats guinea pigs or rabbits often causes death of these animals, but strains vary greatly in pathogenicity

The sulfonamides are of limited value in the treatment of infections caused by *P vulgaris* Streptomycin has proved to be useful in some cases but these organisms readily become resistant to it Chloramphenicol is effective in certain cases

Proteus Morgani

Proteus morgani (Morgan s bacillus) was isolated by Morgan in 1906 from the stools of patients with diarrhea Most strains show the swarming characteristics of the proteus group, usually at lower incubation temperature e g, 20° C but tend to lose this property on cultivation Likewise motility is pronounced at room temperature but it decreases or is lost at 37° C Although Morgan s bacillus is related to *P vulgaris* as shown by its ability to swarm and to decompose urea it does not liquefy gelatin or produce H S It produces indol and ferments for the most part only monosaccharides

P morgani has been isolated on a number of occasions from outbreaks of infantile diarrhea (Neter and Farrar 1943) where it seemed to play an etiologic role, but it is difficult to assess the significance of these findings since it has also been isolated from the stools of normal persons It can cause genitourinary tract infections in man and rarely, purulent lesions of other areas Its role as a potential cause of enteric fever in man has been suspected but not clearly proved Spontaneous epidemics of enteritis due to it have been observed in mice

MISCELLANEOUS GRAM NEGATIVE BACILLI

Pseudomonas Aeruginosa

The pseudomonas group is composed of gram negative rod-shaped motile organisms which characteristically produce a water soluble pigment which diffuses through the medium They occur widely in water and

soil Some species are pathogenic and the type species *Pseudomonas aeruginosa* (*Pseudomonas pyocyanea Bacillus pyocyaneus*) occurs in human feces and in wound and urinary tract infections in man

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The salmonella are gram negative non spore forming motile bacilli which are easily cultivated on ordinary media they characteristically fail to ferment lactose and sucrose The different species are closely

related antigenically and these relationships are used as the main criteria in classification All are pathogenic for man or animals and usually for both *Salmonella typhosa* the cause of typhoid fever is pathogenic only for man while the other salmonella produce disease in man and animals

History

Before the development of modern bacteriology and the isolation of the typhoid bacillus William Budd in 1856 made a strikingly accurate study of typhoid fever outbreaks which led him to believe that the disease was contagious and that the infectious agent was excreted in the feces of patients He believed that contaminated milk and water played a role in the spread of the disease In 1880 Eberth described the typhoid bacillus in tissues of patients and the organism was isolated by Gaffky in 1884 Following this other salmonella were isolated from patients with typhoidlike fevers and it became obvious that a clinical syndrome similar to typhoid fever might be caused by a variety of closely related organisms

Gaertner in 1888 isolated *Salmonella enteritidis* from a patient who died following the consumption of meat contaminated with this organism and shortly afterward Durham and de Noeble de cribed another organism *S typhimurium* isolated from patients suffering with gastroenteritis following ingestion of infected meat Further studies rapidly increased the numbers of organisms identified as having the typical properties of members of the salmonella group

Morphology

Salmonella average from about 2 to 3 microns in length and about 0 6 micron in width but may vary in size under different environmental conditions Young cultures on agar may present a predominance of coccobacillary organisms while filamentous forms are occasionally seen especially in cultures in liquid media With the exception of *S gallinarum pullorum* all strains are motile with peritrichal flagella Salmonella do not ordinarily form capsules when grown at 37° C but most species may give rise to mucoid colonies composed of encapsulated bacilli especially when incubated at temperatures of 20 C or lower Encapsulated strains of *S typhosa* have been described

Cultivation and Biochemical Reactions

Salmonella grow readily on ordinary culture media producing in 24 to 48 hours colonies which average 2 to 3 mm in diameter and are indistinguishable from those of the coliform bacteria The colonies may be circular with a smooth surface and an even edge or flat with an uneven surface and serrated edge On plates incubated at 37° C and then allowed to stand at room temperature they may show a secondary growth of mucoid character around the original colony margin Salmonella in the smooth phase produce a uniform turbidity in broth with a deposit which readily resuspends on shaking They may produce heavy growth under anaerobic conditions The temperature range of growth is from about 10° to 42 C with an optimum at 37° C

The biochemical reactions serve to define the salmonella as a group and also provide an aid in the differentiation of certain species (Table 1) By definition these bacteria do not ferment lactose sucrose or salicin while glucose mannitol maltose and dextrin are fermented with the production of acid and gas except in the case of *S typhosa* and *S gallinarum pullorum* which do not form gas Arabinose xylose trehalose and inositol are useful in the differentiation of certain species and varieties e g *S paratyphi* does not ferment xylose while *S schottmuelleri* does The fermentation of tartrate varies with different species while almost all attack citrate Indol is not produced and gelatin is not liquefied since there are very few exceptions to these two metabolic activities they serve along with the failure to ferment lactose as the most reliable biochemical differential characters

Effects of Physical and Chemical Agents

Most of the salmonella are killed at a temperature of 60 C in from 15 to 20 minutes They may persist under natural conditions for long periods of time as demon

strated in soil and water pollution studies in which typhoid bacilli have been found to survive through an entire winter in frozen soil and for as long as 7 days in well water The resistance of salmonella to certain dyes and chemicals is important since some of these compounds selectively inhibit the coli form organisms while allowing growth of the salmonella Brilliant green in particular in hibits coliform and dysentery bacilli while the typhoid bacillus and other salmonella are resistant to its action Sodium desoxycholate and selenium compounds also inhibit the growth of colon bacilli but not the salmonella under certain conditions Sodium tetrathio nate and sodium citrate favor the growth of salmonella over colon bacilli The selective action of these compounds is used to advan tage in the preparation of media for the isolation of salmonella from feces

Antigenic Structure

The antigenic structure of the salmonella has been studied in great detail by Kauff mann (1937) and White (1926) As a result of their work the various known antigenic components in the cell body and the flagella now constitute the basis of classification The H or flagellar antigens and the O or somatic antigens were described originally as the heat labile and the heat stable anti genic components respectively In general the species of salmonella are divided into groups on the basis of likeness with respect to O or somatic antigens and the species within a group are often differentiated on the basis of differences between their H or flagellar antigens (Kauffmann 1950) Some of these species also possess an envelope antigen called Vi (for virulence) which in hibits agglutination by O antibody

H Antigens and Phase Variation The H antigens are found only in the flagella They are inactivated by temperatures over 60 C and also by alcohol and acids They are probably of a protein nature For serologic testing H antigens are best prepared by adding formalin to young motile broth cul tures This procedure probably fixes the flagella over the surface of the cell in such a way that the somatic antigens are no longer exposed As a result agglutination is de pendent on the anti H antibodies and does not occur or only to a slight degree in anti O sera In sera containing the appropri ate anti H antibodies H antigens character istically flocculate in about 2 hours at 55 C in the form of large fluffy clumps which are easily dispersed

A single species may contain two types of H antigens either of which may predominate in a given instance One of these types is re ferred to as the specific phase or phase 1 flagellar antigen and the other as the group phase or phase 2 flagellar antigen The for mer is shared with only a few other species or varieties of salmonella In contrast the latter may be more widely distributed among several species Either phase 1 or phase 2 may contain one or more flagellar antigenic components Any one culture may consist of organisms entirely of one phase or of organ isms in both flagellar phases Any mono phasic culture usually tends to maintain this characteristic for a number of transfers but is always capable of giving rise to organisms of the other phase especially if the culture is allowed to grow longer than 24 hours This antigen alteration is spoken of as phase variation The transformation from one phase to another in a culture may be induced by growth in a serum containing antibodies against the homologous phase Since the specific phase antigens are not entirely lim ited to one salmonella species but may occur in several the terms phase 1 for the so called specific phase and phase 2 for the group phase have now been adopted Phase variation can be detected only by serologic tests using sera prepared against organisms in phase 1 or phase 2

O Antigens The somatic (O) antigens do not exhibit phase variation and therefore constitute a more dependable basis for classification than the flagellar components (Kauffmann White schema) Somatic anti gens occur at the surface of the cell body (soma) in both motile and nonmotile organ isms as an integral part of the cell wall They are resistant to prolonged heating at 100 C and are not destroyed by alcohol or dilute acid When mixed with sera containing ap propriate anti O agglutinins O (somatic) antigens (prepared from nonmotile bacilli or from bacilli treated with heat or alcohol) are clumped only after long periods of incu

bation e g 6 to 12 hours at 55° C The bacterial aggregates so formed appear as granular masses which cannot be dispersed by shaking

In an antiserum prepared with a motile organism as immunizing agent the anti H and the anti O agglutinins behave independently and the H antibody titer is usually much higher than the O

These O antigens are the endotoxins of the salmonella bacilli and their antigenic specificity is determined by their polysaccharide fraction The major O antigenic components of a given organism are relatively stable but they are related to the presence of temperate bacteriophages and thus are phenomena of lysogenic conversion In recent studies Robbins and Uchida (1962) were able to relate the O antigenic determinants to the presence of specific temperate phages and to show that several phages were specifically associated with the presence of individual hexoses which were antigenic determinants of the polysaccharide complex of the O antigen

Earlier studies (Zinder and Lederberg 1952 Kauffmann 1953) showed that not only O antigens but also H antigens could be transferred from one species to another by transduction with temperate bacteriophages

Vi Antigens Recently isolated strains of typhoid bacilli often fail to agglutinate in usual antiserum Felix and Pitt (1934) showed that this inagglutinability is due to a special somatic component called the Vi or virulence antigen since cultures possessing it were more virulent for mice than ordinary O organisms Vi antigen is thought to be present at the extreme periphery of the cell as an envelope antigen and thus prevents access of anti O agglutinins to their homologous somatic antigens However it is itself a good antigen and Vi antibodies readily cause agglutination of Vi strains It differs from the ordinary O antigens in that its antigenic activity is destroyed at 60 C in 1 hour and by dilute alkali and phenol Following subculture on ordinary media bacilli lose their Vi antigen and become agglutinable in anti O serum A Vi antigen apparently identical with that found in *S typhosa* has also been recognized in *S hirschfeldii*, a member of the Bethesda Ballerup group formerly known as *S ballerup*, and certain strains of *E coli* Other salmonella such as *S schottmuelleri* possess other Vi antigens specific for the species

In 1938, Craigie and Yen discovered bacteriophages active against cultures of the typhoid bacillus containing Vi antigen These agents exhibit an unusual adaptability to the strains of bacteria on which they are propagated resulting in the development of an extraordinary specificity for the particular strain of *S typhosa* Specific Vi phages have thus provided valuable tools for classifying typhoid bacilli and have aided etiologic studies of typhoid fever Later Anderson and Felix (1953) presented evidence that the phage type is an expression of resistance to infection with the typing phages caused by latent phages in the bacteria being tested Strain specific bacteriophages have also been adapted for use in identifying strains of *S schottmuelleri*

Thus another important characteristic of salmonella in addition to their antigenic structure is their content of bacteriophages

Dissociation

In addition to the flagellar phase variations salmonella can undergo a number of alterations related to various antigenic components Motile strains may become nonmotile by losing their flagellar (H) antigens The typical smooth to rough variation occurs frequently and results in the loss of the somatic O antigen and the appearance at the surface of the cell of another antigen which is much less specific and causes the organism to agglutinate spontaneously in saline The smooth to rough change may occur without loss of the Vi or flagellar antigens

The Vi antigen content of any strain of *S typhosa* may vary without affecting its O or H antigens Kauffmann (1935) has described 3 forms in which these organisms may be found (1) the V form which possesses a full quota of Vi antigen and is inagglutinable in O antiserum (2) the VW form in which some Vi antigen may be detected, but which will agglutinate in O antiserum and (3) the W form which contains no Vi antigen Since the H the O and the Vi

TABLE 3 ANTIGENIC STRUCTURE OF SOME OF THE MORE COMMON ENTERIC ORGANISMS

			H ANTIGENS	
GROUP	TYPE	O ANTIGENS	Phase 1	Phase 2
A	*S paratyphosa*	(I) II XII	a	
B	*S schottmuelleri*	(I) IV (V) XII	b	1 2
	S typhimurium	(I) IV (V) XII	i	1 2
C_1	*S hirschfeldii*	VI VII (Vi)	c	1 5
	S choleraesuis	VI VII	c	1 5
	S oranienburg	VI VII	m t	
	S montevideo	VI VII	g m s	
C	*S newport*	VI VIII	e h	1 2
D	*S typhosa*	IX XII (Vi)	d	
	S enteritidis	(I) IX XII	g m	
	S gallinarum pullorum	I IX XII		
E	*S anatum*	III X	e h	1 6

( ) indicate that antigen may be absent

antigens vary independently many dissociant forms can occur

As previously mentioned some salmonella also show a tendency to produce mucoid colonies especially when incubated at temperatures of from 10 to 20 C

KAUFFMANN WHITE CLASSIFICATION

The studies of the salmonella antigens by White and Kauffmann have made it possible to devise a system of classification based on antigenic patterns The species and the varieties have been arranged in groups designated A B C etc according to similarities in content of O antigens and one or more antigenic components are selected as essential for inclusion in each group Each component of the O antigen is designated by the use of a Roman numeral Specific sera to identify O antigens are prepared by absorption technics Table 3 shows how the more important representative salmonella are classified according to the Kauffmann White scheme The members of the various groups based on the O antigen content are further differentiated into species and varieties on the basis of the components of their flagellar antigens The flagellar antigens of phase 1 are noted by small letters and the antigens of phase 2 by arabic numerals

The Kauffmann White system now includes well over 500 varieties many of which are listed as distinct species but it is questionable whether many warrant such recognition since some of the antigenic differences are minor and are now known to be due in many instances to the presence of a variety of temperate bacteriophages in the bacilli Thus most of the closely related organisms might better be considered serologic variants of a single species as has been the practice with serotypes of *Diplococcus pneumoniae* The romance of the salmonella species derived from the association of names with interesting cities famous bacteriologists etc would thus be lost but the classification would be simplified while still retaining its epidemiologic usefulness In this connection it should be pointed out that differences in the ability to ferment one or more carbohydrates exist between strains which apparently are antigenically identical These are designated as fermentative varieties

The scheme of classification based on antigenic analysis is complex but it permits the accurate etiologic diagnosis of enteric infections and facilitates epidemiologic studies

DISTRIBUTION AND RANGE OF PATHOGENICITY

Salmonella fall into 3 groups with respect to their distribution and relationship to human disease

The first group contains those which are

primarily human pathogens and includes *S typhosa*, *S paratyphi*, *S schottmuelleri* and *S hirschfeldii* Of these *S typhosa* is the most important In the United States *S schottmuelleri* is the most common (Seligmann *et al* 1946) occasionally *S paratyphi* is isolated and *S hirschfeldii* is very rare *S schottmuelleri* is also found rarely in animals

The second group is made up of organisms which are primarily pathogenic for animals including birds but occasionally may cause disease in man It contains the majority of the salmonella The relative incidence of these species in human infections varies in different geographic areas Frequently, they are named to designate the area or the city where they were first isolated or to indicate their principal animal host In the United States the following organisms have been most commonly isolated from patients *S typhimurium S choleraesuis S oranienburg S montevideo S newport S enteritidis S panama* and *S anatum* (Bornstein 1943) The relative incidence of infections due to any one species among the reported cases depends in large part on the number of persons involved in the outbreaks studied

In the third group are found those which are known to be pathogenic only for animals or birds This group has rapidly become smaller as more of these species have been found to cause disease in man *S gallinarum pullorum* is one of the most important organisms in this group

Salmonella typhosa (*S typhi Eberthella typhosa Bacillus typhosa Bact typhosum* typhoid bacillus) The typhoid bacillus is found only in man and is the cause of classic typhoid fever In animals by the oral route it will infect only chimpanzees By the intravenous or the intraperitoneal route *S typhosa* in large doses will kill mice but the size of the dose indicates that its invasive powers are not marked and that death in this host is mainly a result of the toxic action of the organisms injected The addition of mucin to the suspension of bacteria enhances the ability of the bacillus to multiply in the mouse and makes it possible to produce death with much smaller numbers of organisms

Salmonella paratyphi (*Salmonella paratyphi A*, *Bacillus paratyphosus A*, *Bacterium paratyphosum A* paratyphoid A bacillus) is found only in man and is one cause of paratyphoid fever

Salmonella schottmuelleri (*Salmonella paratyphi B*, *Bacillus paratyphosus B Bacterium paratyphosum B*, paratyphoid B bacillus) is also a cause of paratyphoid fever in man and in addition produces gastroenteritis Although occasionally it has been isolated from animals it does not cause disease in them under natural conditions

Salmonella hirschfeldii (*Salmonella paratyphi C*) is a common cause of enteric fevers or gastroenteritis in man in Eastern Europe and Asia but is rarely found in the United States It is not known to be a natural pathogen of animals

Salmonella typhimurium (*Salmonella aertrycke Bacterium aertrycke*) is a natural pathogen of rodents causing in mice a typhoidlike disease which has a high mortality It usually produces an acute gastroenteritis in man

Salmonella choleraesuis (*Bacterium suipestifer*) though a natural pathogen for animals may cause an enteric fever or gastroenteritis in man but usually produces localizing processes with or without a septicemia *S choleraesuis* also occurs in the intestine of normal hogs and occasionally in cattle and sheep It is a prominent secondary invader in hog cholera and was once considered to be the etiologic agent until the real cause a filterable virus was isolated Contaminated meat may be a source of infection

Salmonella oranienburg occurs naturally as a cause of epizootics in quail and chickens and frequently is isolated from dried-egg products In man it may produce gastroenteritis enteric fever or septicemia

Salmonella montevideo is found in various animals and fowls (monkeys pigs turkeys chickens etc) and also is isolated from dried-egg products In man it may cause gastroenteritis enteric fever or a septicemia

Salmonella newport is isolated from rats pigs chickens and turkeys as well as from meat and dried eggs It causes gastroenteritis enteric fever or septicemia in man

Salmonella enteritidis (*Bacterium enteritidis*) which resembles *S typhimurium* in many properties was one of the first salmo

nella identified as the cause of disease in man by Gaertner in 1888 It includes several fermentative varieties which sometimes are given separate designations such as *S enteritidis* var *gärtner danyz* or *essen* Some relationship between these varieties and a specific natural host seems to exist Strains have been isolated from horses hogs mice rats ducks and duck eggs In man it causes gastroenteritis more often than other types of clinical manifestations

Salmonella anatum isolated on several occasions from ducks chickens turkeys and dried eggs has also been found in normal pigs and silver foxes In man it usually produces a gastroenteritis

Salmonella derby is found most commonly in turkeys but also occurs in chickens and pigs It is one of the 10 most common salmonella organisms causing gastroenteritis in the United States

Salmonella gallinarum is the cause of fowl typhoid and differs from other salmonella in being nonmotile An important fermentative variant, *S pullorum* is the cause of bacillary white diarrhea in chickens and is found in dried egg products These organisms are commonly regarded as nonpathogenic for man though recent evidence has shown that sometimes *S pullorum* may cause gastroenteritis when ingested in large numbers

From this abridged list of representative salmonella it is apparent that most of the strains that are natural pathogens of animals can also produce disease in man Animal products such as meat milk or eggs are often the vehicles involved in transmission to man These foods may come from infected animal sources or be contaminated by infected animals or man before ingestion

Toxins

The endotoxins of the salmonella are components closely associated with the bacterial body which are released into solution only with autolysis of the cell they are identical with the somatic O antigens of the cell They are heat stable and their toxic effects are neutralized only to a slight degree by immune sera The studies of Boivin *et al* (1933a b) and Henderson (1938) first revealed that these endotoxins are polysaccharide protein lipid complexes The somatic antigens isolated from the various species of *Salmonella Shigella* and *Escherichia* are similar in chemical nature The polysaccharide is responsible for the serologic type specificity of the antigen whereas the protein seems to be antigenically identical in the various salmonella and shigella The component responsible for the toxicity has not been clearly identified though toxic lipids (Westphal and Luderitz 1954) and polysaccharides (Ribi *et al* 1961) have been isolated from the complete complex

The purified somatic antigen complexes elicit the production of O agglutinins bactericidal antibodies and mouse protective antibodies when injected in minute amounts into rabbits or man (Morgan 1941 Favorite and Morgan 1942)

Somatic antigens are highly toxic for animals and man Doses of less than a microgram administered intravenously in man produce a marked febrile response This toxic effect is not completely neutralized by specific immune serum (Morgan 1941) but repeated injection of small quantities leads to an increase in resistance to the toxic effects in animals and man which is non specific in that it is effective against endotoxins that are not antigenically related (Morgan 1948a) This resistance has been called tolerance (Morgan and Favorite 1942) Intradermal inoculation into rabbits and man produces local edema and erythema followed by necrosis Intravenous injection into rabbits produces congestion hemorrhagic extravasation and necrosis in various organs particularly the liver and the bone marrow The latter contains few polymorphonuclear leukocytes The vascular endothelium is damaged and widespread thrombosis similar to the type characteristic of severe typhoid fever in man is produced (Morgan 1943) The changes in the bone marrow and the focal necrosis in the liver are also similar to those observed in fatal cases of typhoid fever Following the intravenous injection in man of minute amounts of the purified somatic antigens the individual develops a chill fever headache malaise and a polymorphonuclear leukopenia These symptoms and the leukopenia are common manifestations of typhoid

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of the organism may account for the persistence of the infection in the presence of bactericidal antibodies in the blood serum and for the failure of therapeutic immune serum to alter appreciably the clinical course of the disease It is probable that the bacilli enter the bloodstream from these cells at the end of the incubation period via the lymphatics and the thoracic duct Then the endotoxins are released in the bloodstream as the bacterial cells are lysed producing some of the symptoms of typhoid fever There is some evidence that the somatic antigens are present in the blood of patients during the acute phase of the disease (Dennis and Saigh 1946)

Typhoid bacilli are found frequently in the spleen and bone marrow and the gallbladder is invariably infected The organisms appear to multiply in the biliary system and the majority of those which are found in the stool at certain stages of the disease particularly in convalescence probably are carried there with the biliary secretions The periodicity of the discharge of the biliary contents into the intestine may account in part for the irregular results in the isolation of *S typhosa* from the feces of any given patient over a period of time

Relapses occur in about 10 per cent of the cases and probably represent a reinvasion of the bloodstream from the local areas of multiplication of the organisms in the lymphoid tissue bone marrow the spleen and the biliary system The mortality rate in typhoid fever in untreated patients is about 10 per cent and death is due to the complications of intestinal hemorrhage or perforation in about 75 per cent of the fatal cases

On postmortem examination the small intestine usually shows extensive areas of ulceration particularly in the area of Peyer s patches and hyperplasia of lymphoid tissue in the intestinal wall Many organisms are present in the lymphoid tissue some within plasma cells The spleen is enlarged The liver shows areas of focal necrosis and organisms are almost always present in the gallbladder Other organs may be involved such as the periosteum bone marrow joints and lungs In patients with signs of meningitis occasionally *S typhosa* has been isolated from the spinal fluid

Recent studies on experimental typhoid fever in man (Greisman *et al*, 1961) have added considerably to our understanding of human infection. It was discovered that it was necessary to administer 5×10^8 organisms to the volunteers by mouth to ensure infection though the severity of the illness produced varied from person to person Such subjects developed tolerance to the toxic effects of endotoxin early in convalescence but this resistant state disappeared after 3 months Following recovery, several volunteers had relapses of their infection with bacteremia at times when they had high titers of circulating antibodies for *S typhosa*

It has also been possible to infect chimpanzees by mouth with several billion typhoid bacilli with the production of a febrile illness resembling mild typhoid fever in man and in which the pathologic findings were essentially indistinguishable from those seen in man (Edsal *et al* 1960)

Other Enteric Fevers

Enteric fevers caused by salmonella other than *S typhosa* have a shorter incubation period from 1 to 10 days and, with the exception of some of the cases due to *S paratyphi S schottmuelleri* and *S hirschfeldii* are milder and less typical than typhoid fever Fever and malaise are the dominating symptoms and last from 1 to 3 weeks Blood cultures are often positive early in the disease while stool cultures may be negative for 1 or 2 weeks Rose spots are rare The postmortem findings may or may not be similar to those of mild typhoid fever *S schottmuelleri* is a common cause of enteric fever in the United States

Gastroenteritis

Following the consumption of contaminated food gastroenteritis caused by salmonella occurs after an incubation period of from 8 to 48 hours This short interval suggests that large numbers of the organisms are usually ingested The onset is nearly always sudden and may be characterized by headache chills and often abdominal pain Nausea vomiting and diarrhea follow with a rise in temperature prostration develops which lasts from 1 to 4 days The disease is more severe in infants and young children Blood cultures are usually negative but frequently organisms can be isolated from the

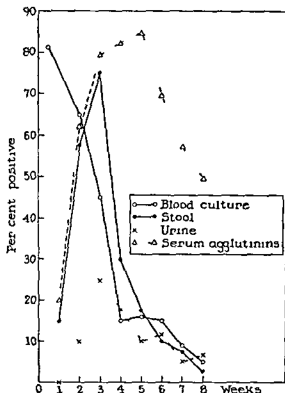


Fig 1 Results of serum agglutination test and of blood stool and urine cultures of patients during the course of typhoid fever

fever in man and it is likely that they are produced by the release of the endotoxins from the typhoid bacilli present in the infected subject

A local intradermal injection of the somatic antigens followed after an interval by an intravenous injection results in the local hemorrhagic changes in the skin characteristic of the Shwartzman reaction

Pathogenesis

Salmonella infection is almost always due to the ingestion of contaminated materials the organisms enter the tissues from the intestine via the lymphatics Since the number of organisms ingested may affect the length of the incubation period the latter may vary considerably in different individuals

There are 3 main types of clinical manifestations of salmonella infection namely enteric fever, gastroenteritis and a localizing type with foci in one or more organs accompanied by septicemia

Typhoid Fever

Among the enteric fevers the classic example is typhoid fever The typhoid organism unlike other salmonella produces this clinical manifestation only in the human host and in the chimpanzee The incubation period extends from about 7 to 14 days The onset is insidious often beginning gradually with malaise, anorexia and a headache This usually is followed by the appearance of a fever which rises in a steplike manner to an average of 104° F, with a pulse rate that tends to be slow in comparison with the height of the fever Nosebleeds may occur at this stage of the disease During the first week the patient usually is prostrate and may have diarrhea though constipation is even more common and either is usually accompanied by abdominal tenderness and distension At this time the patient may also have a cough and bronchitis In the first or the second week rose spots frequently appear splenomegaly is common and the temperature remains elevated In the more severe cases, as time passes the patient may become delirious and show the so called typhoid state for which the disease was named After the 3rd week the temperature curve shows morning remissions and returns to a normal level by a gradual lysis A leukopenia is present in most cases characterized by a relative decrease in the number of polymorphonuclear leukocytes and an absence of eosinophils

Blood cultures are often positive in the 1st and the 2nd weeks and less frequently during the 3rd week stool cultures may be positive from the beginning and often remain positive until convalescence has been completed Organisms are often found in the urine during the second and the third week and may be excreted for a considerable period after convalescence (Fig 1) Cultures of the bone marrow may show typhoid bacilli when blood cultures are negative Organisms may also be found in the rose spots

The typhoid bacillus gains access to the body through the alimentary tract where it probably multiplies during the incubation period in the lymphoid tissue of the wall of the small intestine and the regional lymph nodes Later organisms are often found here in the plasma cells The intracellular position

the blood but that the bacilli which are known to occur intracellularly in the spleen the gallbladder bone marrow and the lymphoid tissue of the intestine are protected from their action These bacilli multiply within the cells and release the somatic endotoxins which seem to produce tissue damage and the symptoms of general toxemia Since the toxicity of these substances is only reduced and not completely neutralized by antibodies they may produce their injurious effects in the presence of circulating antibody (Morgan 1941) Therefore humoral antibodies appear to have a limited role in immunity to typhoid fever and the decisive factor in recovery may be the development of an increased capacity of the fixed phagocytic cells to destroy the bacteria The persistence of resistance after antibodies have disappeared from the blood tends to support this hypothesis

Another factor of possible importance in recovery from the disease may be the development of an increased tolerance to the toxic effects of the somatic antigens not dependent on the presence of antibodies (Neva and Morgan 1950) In man increasing doses of typhoid somatic antigen may be given intravenously without corresponding increases in the severity of successive reactions (Favorite and Morgan 1942) This tolerance does not seem to be correlated with antibody titer since relatively small amounts of toxin may produce toxic effects in the presence of high levels of circulating antibody Furthermore tolerance disappears fairly rapidly after the injections of toxin are discontinued while the antibody levels remain elevated for much longer periods This type of tolerance may involve a change in the functional activity of the reticuloendothelial system, providing for a more rapid disposal of foreign material (Beeson 1946) It is not specific for a particular somatic antigen but appears to extend to the chemically and toxicologically related but immunologically distinct endotoxins of other gram negative organisms (Morgan 1948a b)

In addition to the earlier observations (Neva and Morgan 1950) regarding the development of tolerance to endotoxins in natural enteric fevers caused by salmonella the studies on experimental typhoid fever

TABLE 4 MORTALITY FROM TYPHOID FEVER

PERIOD	UNITED STATES (Rates per 100 000)	UNITED STATES ARMY (Rates per 100 000)
1906-1910	25 6	26 37
1911 1915	16 6	3 24*
1916 1920	11 1	5 08†
1921 1925	7 6	0 4
1926-1930	5 1	1 19
1931 1935	3 5	0 59
	93 CITIES‡ IN THE UNITED STATES	UNITED STATES ARMY IN THE EUROPEAN THEATER§
1942 1945	0 25	0 06

* 1911—Compulsory immunization introduced into U S Army

† 1916—Mexican Border Service 1917 1919 World War I

‡ Total population of 38 060 662.

§ Army had maximum strength of 3 064 562 in this theater

have shown clearly that significant tolerance is present during convalescence from these carefully studied infections in human volunteers (Greisman *et al* 1961)

IMMUNIZATION

Within a few years after the isolation of *S typhosa* suspensions of killed bacilli were injected into human beings for the purpose of immunization Adoption of this procedure in military personnel was accompanied by a marked decrease in mortality due to typhoid fever (Table 4) Although available data tend to support the view that vaccination significantly reduces the incidence of this infection it is often difficult to assess the results since general conditions of sanitation began to improve both in military and civil practice at about the same time that vaccination was introduced In consequence the use of the comparative incidence of typhoid fever during consecutive periods of years as an indication of the efficacy of vaccination is invalid unless the sanitary conditions are known to be identical in these successive periods For this reason a strictly controlled field trial was organized recently in Yugoslavia by the World Health Organization and it was

feces and occasionally from the vomitus *S typhimurium* is the organism most commonly isolated from cases with salmonella gastroenteritis in the United States

Septicemias

In the septicemias caused by salmonella the invasion of the bloodstream is evidenced by the high remittent fever and positive blood cultures Intestinal involvement is usually absent in adults, although in children the septicemia may occur as a complication of gastroenteritis Organisms may localize in any tissue of the body and may produce local abscesses in the perineal and the pelvic regions cholecystitis, pyelonephritis, endocarditis, pericarditis meningitis arthritis or pneumonia *S choleraesuis* is one of the most common organisms found in this particular type of infection The mortality in salmonella septicemia averages about 5 per cent but may reach 20 per cent in infections with *S choleraesuis* (Seligman *et al* 1946)

Every *Salmonella* strain is potentially capable of producing any of these 3 clinical types of infection though *S paratyphi* and *S schottmuelleri* tend more frequently to cause the enteric fever picture This potentiality may be manifested in the clinical course of disease in a single patient such as onset of illness as acute gastroenteritis and after temporary improvement the development of an enteric fever due to the same organism (e g *S schottmuelleri*) *S typhimurium* and *S enteritidis* are found predominantly in acute gastroenteritis while *S choleraesuis* is found mostly in the septicemic type of infection In all instances infections produced by salmonella are more severe in infants and children than in adults Subclinical infections in which symptoms are absent or so mild as to cause no disability often occur with salmonella other than *S typhosa* and organisms are found in the feces In some instances especially in gastroenteritis more than one species of salmonella may be isolated from the stool of a single patient (Saphra and Winter 1957)

The organisms are frequently excreted in the feces during convalescence from salmonella infections or after subclinical infections and occasionally for months thereafter When the infection is due to *S schottmuelleri* 20 per cent of the patients show positive fecal cultures 3 months after the disease The proportion of patients with positive cultures begins to drop off rather rapidly after this to reach a level as low as 5 per cent at the end of the fourth month though a few become chronic carriers

Carriers

In some instances the salmonella may establish themselves in the tissue of the host to produce a more or less permanent carrier state after the recovery from an acute infection This is most likely to occur following typhoid fever, where about 3 per cent of the cases are found to be excreting *S typhosa* in their stools over a year after recovery from the disease In this instance typhoid bacilli are present in the gallbladder or, less frequently in the kidney tissue where they multiply, they reach the feces or urine sporadically

The carrier state in human beings is observed less frequently with *S paratyphi* and *S schottmuelleri* than with *S typhosa* and its duration is much shorter In a study of a large number of healthy carriers (Seligman *et al*, 1946) 28 different salmonella types were isolated with *S schottmuelleri S typhimurium, S oranienburg S montevideo S newport, S panama* and *S anatum* being the most common Many of these carriers were contacts of cases and had exhibited no clinical symptoms of infection

Immunity

An attack of typhoid fever usually confers immunity though second attacks have been reported Recovery from the disease is associated with the appearance in the blood of agglutinins and bactericidal antibodies for *S typhosa* These antibodies reach appreciable levels during the second and the third weeks of the disease at a time when the typhoid bacilli are known to disappear from the bloodstream However, antibodies may be present during the acute phase of the disease in relapses and at the time of progression to fatal termination (Gay 1918) These facts strongly suggest that known antibodies are not the sole factor in recovery

It seems probable that the circulating antibodies clear the extracellular organisms from

to 90 per cent of cases during the first week (Fig 1) Blood cultures are usually negative in salmonella gastroenteritis

Organisms may be isolated from the feces in typhoid fever or in salmonella gastroenteritis during the acute stage of the disease and for a variable period thereafter In typhoid fever the number of positive fecal cultures may increase during the second week Organisms may also be isolated from the vomitus or from the contaminated food ingested in gastroenteritis In *S typhosa* infection as many as 25 per cent of the cases have positive urine cultures in the later stages of the disease Salmonella may be found in the urine of patients with the septicemic type of infection when the organisms have localized in the kidney

It has been found that several different species of salmonella may be isolated from the stools of patients involved in a single outbreak of salmonellosis and that 2 or 3 species may be found in the same patient (Saphra and Winter 1957)

Isolation of salmonella from the feces presents a special problem because of the huge number of normal fecal organisms (It is important to culture the fecal specimen as soon as possible to prevent overgrowth by the normal fecal flora a direct rectal swab may be a useful adjunct in obtaining a fresh specimen) A variety of materials have been added to special media to aid in the isolation of intestinal pathogens They fall into several categories (1) agents which inhibit the growth of normal intestinal bacteria e g dyes like brilliant green selenium salts and bile salts (2) agents which favor the growth of salmonella over the coliform organisms e g sodium tetrathionate and sodium citrate (3) substances which give to salmonella colonies distinguishing characteristics e g lactose with necessary dye indicators to color the fermenting colonies of the coliform group or sulfite which is reduced to sulfide by many salmonella and gives a black color to the colony in the presence of iron salts

The fresh stool specimen should be streaked immediately on a selective medium e g SS agar or sodium desoxycholate citrate agar and on a nonselective medium such as EMB or MacConkey The nonselective medium is included to pick up the unusual species of salmonella that do not grow on the highly selective SS medium. For use in the isolation of *S typhosa* the bismuth sulfite selective medium is of particular value At the same time the specimen should be inoculated into an enrichment medium which allows the pathogens to multiply and inhibits *E coli* e g tetrathionate or selenite broth After incubation for 8 to 12 hours this enrichment culture is streaked on selective and nonselective agar media Suspected colonies are subcultured and identified by biochemical reactions and by agglutination tests with specific absorbed sera Further species identification of salmonella can be obtained by sending the culture to one of the Salmonella Centers

Serologic Diagnosis Agglutinins for the causative organisms usually can be demonstrated in the circulating blood of patients from 1 to 2 weeks after the onset of typhoid and enteric fever septicemia or gastroenteritis of the more severe types In mild diarrheas the agglutination reaction may not become positive until after the symptoms have subsided or in some instances it may remain negative Tests should be carried out for both O and H agglutinins but O agglutinins appear to be of more significance since some cases fail to develop H agglutinins Moreover O agglutinins are of more diagnostic value because they disappear more rapidly than H agglutinins following vaccination Finally O antigen relationships among the various salmonella broaden the range of the test for the initial detection of infection

The antigens used in the agglutination test should be so composed as to give an adequate coverage for all of the principal salmonella groups occurring in the particular geographic area e g O antigens of groups B C and D and A where indicated After this preliminary screening further testing can be carried out to give definite diagnosis of the species involved In the United States *S typhosa S schottmuelleri* and *S choleraesuis* are commonly used for screening agglutination tests It is not necessary to include a Vi antigen for the diagnosis of typhoid fever since Vi agglutinins rarely appear in the blood of patients in the absence of H or O agglutinins Also Vi agglu

shown that a phenolized vaccine containing O but not Vi antigen was effective in reducing the rate of infection thus demonstrating the effectiveness of immunization but raising the question of the role of the Vi antigen in protection against typhoid fever (Cvjetanovic 1957) However recent tests have shown that a vaccine with a high content of Vi antigen may be more effective than one containing predominantly O antigen In another study by Callender and Luippold (1943) in which a standard vaccine had been used for the immunization of a group of men who subsequently were exposed to a common contaminated water supply, the incidence of typhoid fever among the nonimmunized group was 7 per cent as compared with 1 1 per cent in the vaccinated group These results suggest that the protection conferred by vaccination is only relative since cases did occur in immunized groups Since the infecting dose may be very large in some instances the immune mechanism may be overwhelmed thus explaining the failure of the vaccine to give complete protection under all conditions (Syverton *et al* 1946)

In experimental typhoid fever in chimpanzees bacterial vaccines were found to be effective in protecting against infection whereas purified Vi antigen produced some immunity and purified O antigen induced no discernible protection (Gaines *et al* 1961) Thus the use of purified antigens for immunization requires further study and the relative importance of Vi and O antigens needs to be clearly elucidated

The vaccine commonly used consists of a saline suspension of typhoid organisms in the smooth phase killed by heating for 1 hour at 56° C The suspension is standardized to contain 1 billion organisms per ml Tricresol or an organic mercurial is added as a preservative

Grinnel (1932) studied the immunizing potency of the various dissociants of the typhoid bacillus and found that vaccines prepared from rough strains had little value This was confirmed by the findings (Felton and Wakeman 1937) that the O somatic antigens which are lost in the S → R dissociation are the essential immunizing constituents of salmonella Recently recognition that the Vi antigen is destroyed by heating has led to the use of an alcohol inactivated vaccine in which the Vi antigen is preserved However as mentioned above it has not yet been demonstrated that the Vi antigen plays an essential role in protection.

Since the O antigens can now be prepared in relatively pure form they have been used in experimental immunization in man In very small amounts, they produce somewhat higher circulating antibody titers than the equivalent quantity of whole bacterial vaccines with less unpleasant toxic reactions (Morgan, 1945)

During periods when sanitation breaks down a wider range of protection may be desired, and typhoid vaccine is often supplemented by the addition of suspensions of heat killed *S paratyphi* and *S schottmuelleri* to form T A B vaccine In certain geographic areas this vaccine includes *S hirschfeldii* (T A B C vaccine) These vaccines are given in the form of 3 injections of 0 5 to 1 0 ml subcutaneously and reinoculation with 0 5 ml every 3 years The administration of 0 1 ml intracutaneously as a booster dose produces less fever and undesirable reactions yet it appears to give adequate antibody titers It has been suggested that the intracutaneous route might be used also for primary immunization Administration of vaccines by the oral route has not been shown to elicit an adequate antibody response

The value of antityphoid immune serum used prophylactically in individuals known to have been exposed to typhoid fever has not yet been established

Diagnosis

The diagnosis of salmonella infection depends on isolation and identification of the causative agent (e g from blood feces or urine) or on the demonstration of a rise in titer of specific circulating antibodies for a given organism

Isolation of the organism from the circulating blood is the most conclusive method of bacteriologic diagnosis It is most likely to be successful in the earliest phases of the disease and in the enteric fever or septicemic types of clinical disease In typhoid fever positive blood cultures are obtained in 80

contaminated food in which the organisms often multiply before ingestion

Water may be contaminated with infected feces by cross connections between water and sewage pipes seepage of surface water into wells or surface contamination of shallow wells Epidemic outbreaks of typhoid fever involving entire communities may follow a breakdown of the water purification system during a flood for example Outbreaks involving fewer individuals may be caused by contaminated milk not pasteurized or more rarely contaminated after pasteurization In these instances the distribution of cases parallels that of the delivery of the milk Oysters and shell fish may also cause smaller outbreaks when taken from water contaminated with sewage

Endemic typhoid fever and infections with other salmonella are now much more common than epidemic typhoid fever in parts of the world where sanitary measures are applied to protect water and food supplies In these instances infection usually is due to contamination of food by a human being or an animal excreting the organisms or to the ingestion of the improperly cooked meat of an infected animal or of foods containing contaminated egg products In some instances flies carry infected excreta to the food or even become infected themselves with the bacilli and deposit them on the food Milk and dairy products such as cream custard ice cream and cheese have been involved in the spread of typhoid fever and other salmonella infections The bacilli can grow in foodstuffs at warm temperatures without producing any detectable changes in appearance or taste to reveal their presence Among foods prepared from tissues of animals infected with salmonella bacilli are meat products like sausage and luncheon meats which are often insufficiently cooked Eggs are often implicated and positive salmonella cultures have been obtained in as many as 30 per cent of a series of preparations of powdered eggs most of which contained *S pullorum* although *S oranienburg* *S typhimurium* and *S montevideo* were also found Ducks are known to harbor *S typhimurium* and *S enteritidis* which have been found in their eggs

Less than 5 per cent of recognized clinical cases of typhoid fever become chronic carriers and continue to excrete *S typhosa* in their stools and in some instances in their urine for years They constitute the main source of infection in this disease although ambulant cases also spread the organisms Since over 90 per cent of typhoid carriers show Vi agglutinins in their serum this provides a valuable screening test for their detection Once proved to shed bacilli in their urine or feces these persons must be isolated and kept under medical supervision until rid of the carrier state by procedures such as cholecystectomy or drug therapy Disappearance of Vi agglutinins suggests that the carrier state has been eradicated but this is proved only by repeated negative fecal and urine cultures As the incidence of typhoid decreases the number of carriers is reduced sharply thus drying up the reservoir of infection

Chronic carriers who excrete organisms for over a year are found much less often with *S paratyphi* and *S schottmuelleri* and even more rarely with other organisms such as *S typhimurium* With other salmonella the most important sources of infection are the temporary convalescent carriers who may continue to excrete the bacilli for from 4 to 16 weeks after their illness and the ambulant, subclinical cases who excrete the organisms in their stools In any outbreak there may be a large number of these apparently normal contacts who are excreting the bacilli and therefore spreading the infection

Control Measures

The control of salmonella infections is directed at (1) the detection and the elimination of the modes and the vehicles of transmission (2) the elimination of the sources of infection and (3) measures to increase the resistance of the susceptible host The first two aspects of control are the most important and usually are relied on entirely except under circumstances in which control of the environmental factors become more difficult as in time of war when the third aspect may assume a more critical role

In the case of typhoid fever elimination of sources of infection is largely a question of detection and supervision of the chronic human carrier This measure is also valuable

tinins may not be detectable in the serum of patients known to have typhoid fever The detection of agglutinins in the feces of patients (Harrison and Banvard, 1947) has been suggested as a diagnostic method

In the Widal or agglutination test, the demonstration of a rising titer of specific agglutinins is accepted as definite evidence of infection with the particular salmonella strain However, if only a single specimen is available, an O agglutinin titer of more than 1 50 during the first 10 days of illness is considered strong presumptive evidence if the patient has not been vaccinated within 2 years In a patient with a history of previous inoculation with typhoid vaccine, the fact that H agglutinins tend to persist for a number of years following immunization, while O agglutinins fall in 6 months and usually disappear in about a year makes the O agglutinin titer more valuable in diagnosis The common concept that such agglutinins will rise in titer during any febrile illness due to a nonspecific anamnestic reaction has been found to be erroneous (Koomen and Morgan 1954) However several other factors should be considered in the interpretation of a single agglutination titer The level of agglutinins in the serum of normal persons in the particular geographic area is important and in all instances the stage of the disease at which the serum specimen was taken should be noted Since antigen preparations differ in their sensitivity to the agglutinating action of sera, the activity of each new antigen should be evaluated with a standard serum The test for O agglutinins can be made much more sensitive by adsorbing O antigens to the surface of human type O erythrocytes These red cells when added to serum containing the anti-O antibodies agglutinate to high titer (Neter *et al* 1956)

Treatment

The treatment formerly used in typhoid fever or other salmonella infections was mainly supportive and aimed at maintaining the fluid balance and the nutritional state of the patient More recently several specific therapeutic agents have been employed The sulfonamide drugs apparently have had some beneficial effect on certain salmonella infections but their use in typhoid fever has been disappointing Combined therapy using sulfonamides with larger than ordinary doses of penicillin has given some evidence of therapeutic value Streptomycin has had a limited trial in typhoid fever and in other salmonella infections without any clearly beneficial results, in spite of the fact that the organisms are sensitive to the action of the drug in vitro (Keefer, 1946) Streptomycin given orally markedly reduces the number of typhoid bacilli as well as of coliform organisms in the stools However, the bacteria reappear when the therapy is discontinued

Chloramphenicol is effective in the treatment of typhoid fever (McDermott Knight and Ruiz Sanchez 1949), though patients do not become afebrile until about the fourth day after treatment is started and may become carriers in spite of adequate therapy However, chloramphenicol therapy of other salmonella infections has been less successful

The intracellular location of the typhoid organisms is also thought to account for the relatively slow response of this infection to antibiotic therapy In a model system in vitro where *S typhosa* bacilli were studied inside mouse fibroblasts, chloramphenicol promptly inhibited their multiplication but viable bacilli remained within the cells for several weeks (Showacre *et al* 1961 Hopps *et al* 1961)

Epidemiology

The source of all salmonella infection is the reservoir of organisms living in the tissues of human beings or animals Infection occurs through food, milk or water contaminated with infected feces or urine or by the actual ingestion of the infected animal tissues The hosts which harbor the organisms may be clinically recognized cases or sick animals subclinical cases or carriers The two latter groups are the most important, since they are usually unrecognized Infection with most salmonella apparently requires the ingestion of large numbers of organisms but in the case of *S typhosa* relatively few bacilli are sufficient to cause typhoid fever This difference in infectivity probably accounts for the fact that lightly contaminated material like water or shellfish can be the source of typhoid infection while most other salmonella infections are caused by heavily

dysentery The clinical and epidemiologic descriptions furnished by Hippocrates in the same century suggest that the disease was well known in Greece and that the bulk of it was probably of bacillary rather than amebic origin Until the recent advent of effective sanitation the conditions associated with military operations have been particularly conducive to the spread of enteric disease and it is not surprising that outbreaks of dysentery have been a frequent accompaniment of military campaigns In recent years the Gallipoli campaign of World War I, the operations in the Mediterranean and the Pacific Theaters of World War II and the experiences in prisoner-of war camps during the Korean War have furnished invaluable clinical laboratory and epidemiologic data to students of the disease In civil life jails and asylums have long been notorious for outbreaks of dysentery overcrowding poor sanitary facilities and low standards of personal hygiene appear to have been the common factors

In spite of the many excellent clinical accounts of dysentery which have been published since the time of Herodotus it was not until the closing years of the 19th century that the bacillary and the amebic varieties were separated on clinical epidemiologic and etiologic grounds The latter disease occurs sporadically rather than in epidemics pursues a chronic rather than an acute course is frequently complicated by hepatic abscesses if specific therapy is not instituted promptly and presents the typical pathologic findings of shallow undermining ulcers in the large bowel and an intestinal exudate which is mononuclear in character The causative agent *Entamoeba histolytica* probably was discovered as early as 1859 and by 1890 it was firmly established as one of the etiologic agents of the disease syndrome

The first successful attempt to incriminate a specific bacterial agent as a cause of dysentery resulted from the work of Shiga in connection with an outbreak of dysentery in Japan in 1896 He isolated the organism now known as *Sh dysenteriae* type 1 from the feces and the intestinal walls of patients suffering from clinical dysentery Further proof was furnished by the finding of specific agglutinins against the organism in the blood of patients suffering convalescing or recently recovered from the disease but not in the blood of healthy individuals (Shiga 1898) Two years later the first type of *Sh flexneri* was isolated by Flexner in the Philippines in similar fashion from the feces of patients suffering from dysentery The first adequate description of *Sh sonnei* was given by Sonne of Denmark in 1915 *Sh dysenteriae* type 2 was isolated by Schmitz in a Roumanian prisoner-of war camp during an outbreak of dysentery in 1917 Boyd isolated the first strain of *Sh boydii* in India about 1930 *Sh dysenteriae* types 3 through 7 represent the so-called Large Sachs group of dysentery bacilli Although the existence of such a group was suspected as early as 1919 much of our information concerning them comes from the more recent work of Sachs and others in India

Morphologic and Biochemical Characteristics

The shigella organisms are slender gram negative rods approximately 1 μ by 0 5 μ They resemble other enteric bacteria but are frequently so short on primary isolation as to resemble coccobacilli They are nonmotile and nonsporulating and no true capsule has been identified

Shigella bacilli are aerobes and facultative anaerobes like other enteric bacilli and grow best at a temperature of approximately 37° C Their nutritional requirements while not completely defined are relatively simple and the ordinary culture media will support their growth Their ability to grow in the presence of various bile salts in contrast with some strains of coliform bacilli is made use of in the design of selective media (e g SS agar) for their isolation from the gastrointestinal tract They do not liquefy gelatin or produce hydrogen sulfide they are not able to utilize citrate All members of the group fail to produce acetyl methyl carbinol but are able to reduce nitrates to nitrites Some species produce indol There is evidence that in the case of *Sh flexneri* 3 at least the tricarboxylic acid cycle may function as the pathway of terminal respiration (Pan *et al* 1957)

As in the case of other nonsporulating organisms their resistance to physical and

in the prevention or the spread of other salmonella infections In all instances the in fected patient must be recognized isolated and his excreta carefully disposed of Bac teriologic examinations should be made of the stools in all cases of diarrhea and all salmonella infections should be reported The detection and the isolation of the ambulant cases in an outbreak is a valuable adjunct in control when this procedure is feasible Ideally any individual yielding positive cul tures of feces or urine should be isolated until several successive negative cultures have been obtained

Adequate inspection of animals slaught ered for meat is of value in limiting the con sumption of meat from sick animals How ever if the disease is not active the detection of infected animals may be very difficult Inspection and supervision of other animal food products also serve to reduce con tamination

Spread of the infection can be halted by methods directed at the vehicles and the modes of transmission Modern sanitation methods such as proper sewage disposal se lection of unpolluted sources of water supply with its subsequent filtration and chlorination and the pasteurization of milk have been largely responsible for the dramatic decrease in the incidence of typhoid fever

The contamination of food may be pre vented by the exclusion as food handlers of persons with diarrheas and by the elimination of rodents and flies from premises where food is prepared *Careful handling of food after* preparation adequate refrigeration of un cooked foods adequate cooking of meats eggs and egg products all serve to reduce salmonella infection

Active immunization with appropriate vac cines should be regarded as an emergency measure to be used under conditions of great exposure for example among the staffs of contagious disease hospitals among soldiers or travelers in countries where the diseases are still endemic due to lack of sanitary de velopments or when sanitation breaks down as in catastrophes or periods of war Vac cination is a poor substitute for sanitary control since there is no evidence that it alone can stamp out intestinal diseases though it seems to reduce the incidence and the fatality of salmonella infections Cases do occur among vaccinated individuals (Syverton *et al* 1946) especially when the infective dose is large In the prisoner of war camps in World War II vaccination ap- parently was effective in aiding in the control of disease until the camps became severely overcrowded Prophylactic vaccination dur ing an epidemic is of value mainly in pre venting secondary cases among contacts of infected individuals If the outbreak is ex plosive as in the case of a waterborne epi demic of typhoid fever where the source can be discovered and controlled early immuni zation of the entire community is not neces- sary since it can have no effect on individuals in the incubation period of the disease and is unnecessary for those who have escaped infection with the exception of those in direct contact with the patients (Topley 1938)

THE SHIGELLA AND BACILLARY DYSENTERY

The shigella organisms are nonencap- sulated nonmotile slender gram negative rods which are facultative anaerobes growing best under aerobic conditions They ferment various carbohydrates without the produc tion of gas and vary in the fermentation of mannitol which is used as an important characteristic in their classification One species (*Shigella sonnei*) ferments lactose after incubation for 2 days or more Some of *the shigella share antigens in* common with other genera of the family *Enterobacteri aceae* and also possess distinctive antigens which are useful in their classification In contrast with the widespread distribution of salmonella and coliform organisms the shigella are found only in the gastrointestinal tracts of primates and thus the only im portant source of infection in man is the human host In man these organisms cause bacillary dysentery

History

The clinical entity of bloody or mucous diarrhea accompanied by straining and tenes mus was recognized as long ago as the 4th century B C Herodotus ascribes the defeat of the Persian Army in 380 B C in part to

dysentery The clinical and epidemiologic descriptions furnished by Hippocrates in the same century suggest that the disease was well known in Greece and that the bulk of it was probably of bacillary rather than amebic origin Until the recent advent of effective sanitation, the conditions associated with military operations have been particularly conducive to the spread of enteric disease and it is not surprising that outbreaks of dysentery have been a frequent accompaniment of military campaigns In recent years the Gallipoli campaign of World War I the operations in the Mediterranean and the Pacific Theaters of World War II and the experiences in prisoner-of war camps during the Korean War have furnished invaluable clinical laboratory and epidemiologic data to students of the disease In civil life jails and asylums have long been notorious for outbreaks of dysentery overcrowding poor sanitary facilities and low standards of personal hygiene appear to have been the common factors

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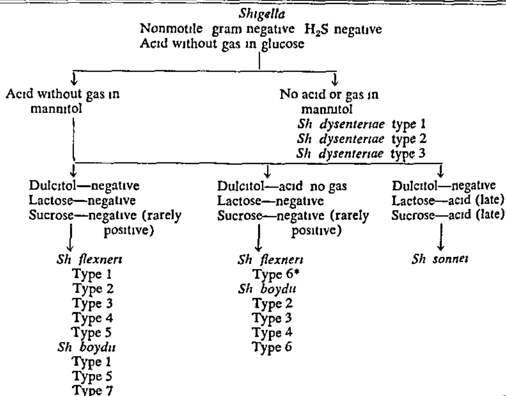
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TABLE 5 BIOCHEMICAL SCHEMA OF THE SHIGELLA



* Occasional strains of this type have been recovered which produce acid and gas in mannitol and glucose

chemical agents is not remarkable A temperature of 55° C sustained for 1 hour will kill them as will exposure to 1 per cent phenol for 30 minutes However they may survive in sea water for at least 3 days and for a considerably longer period in the dry state particularly if they are kept in the dark Under natural conditions in the stool their survival appears to be short presumably due to bacteriophage action or because of their sensitivity to the acidity produced by the growth of other organisms Thus it is important to culture stool samples as promptly as possible

Individual species of shigella ferment a variable number of carbohydrates with the production of acid but with two exceptions no gas Although some variation in this ability occurs among strains of a given type or species, the fermentation reactions are sufficiently constant and characteristic to be of use in their identification and classification All ferment glucose and none salicin adonitol or inositol On the basis of their ability to ferment mannitol they may be divided into 2 primary groups (1) *Sh sonnei Sh boydii* and *Sh flexneri* (with the exception of a few strains belonging to type 6) ferment mannitol (2) in the non mannitol fermenting group fall all 7 types of *Sh dysenteriae* Lactose is fermented by *Sh sonnei* and rare strains of *Sh dysenteriae* 1, however, the fermentation is characteristically slow and is not apparent for several days In the case of *Sh sonnei* at least this property is due to the development of lactose fermenting papillae on the original lactose negative colonies grown out on primary isolation (Glynn and Starkey, 1939) With the exception of these species, the members of the genus *Shigella* resemble the other enteric pathogens in their inability to ferment lactose Two strains of *Sh flexneri* 6 form exceptions to the rule that dysentery bacilli are anaerogenic The so-called Newcastle strain may produce a small quantity of gas in both glucose and dulcitol while the closely related Manchester strain may produce gas in man

nitol as well A useful point in the differentiation of *Sh dysenteriae* 1 and 2 is the ability of the latter to produce indole as compared with the failure of the former to do so Of the less common types of *Sh dysenteriae* 5 and 7 produce indole while 3 4 and 6 do not While these various biochemical reactions are of considerable usefulness in the tentative differentiation and identification of species and types, the final identification of the various shigella depends on the study of the antigenic structure

The biochemical reactions are summarized in Table 5

Antigenic Structure

The antigenic pattern of the shigella is complex Most species tend to be antigenically heterogeneous some much more so than others Serologic overlapping between different species occurs because of the presence of common group antigens Furthermore certain of these group antigens are found in other enteric bacilli The fact that some strains of shigella may be rendered more agglutinable by boiling or other processes suggests that in addition to group- and type specific antigens these organisms may contain K (or envelope) antigens as well An additional source of trouble is the occasional instance in which there is a lack of correlation between serologic and biochemical characteristics In such a case antigenic structure usually is accepted as the final criterion for the purposes of classification

Sh dysenteriae 1 (originally isolated by Shiga) and 2 (originally isolated by Schmitz) are antigenically homogeneous That is an antiserum prepared against a strain of a given type will agglutinate all other strains of the same type to approximately the same titer but will not agglutinate strains belonging to other types or species *Sh dysenteriae* 3 4 5 6 and 7 comprising the so-called Large Sachs group all possess type specific antigens by which they may be identified Some sharing of group antigens with certain coliform organisms has been reported and the fact that boiled cultures usually produce stronger agglutination reactions suggests the presence of K antigens

Sh flexneri presents a complicated picture which has given rise to several confusing systems of classification and terminology Madsen (1949) reviewed the work of earlier investigators and reported the results of his own extensive observations Six type specific antigens (comprising types 1 to 6) have been recognized by means of absorbed sera In addition to the type specific antigen limited to the type in question each type contains 1 or more group antigens which may be present in other types as well At least 4 of these group antigens responsible for the numerous cross reactions characteristic of this group have been identified They permit the division of the individual types comprising this group into subtypes In the case of 2 well known strains (X and Y) no type specific antigen has been identified Therefore these strains are usually considered to be degraded variants which have lost the type specific antigen

The 11 types belonging to *Sh boydii* present a simpler antigenic pattern in that no significant group antigen or antigens have been recognized In his pioneer work Boyd (1940) recognized 6 specific types on the basis of distinctive type antigens while a 7th type was identified by several independent investigators during World War II Four additional serotypes have been recognized more recently (Edwards and Ewing 1955) The absence of group antigens largely eliminates confusing cross reactions and the typing of the individual members of this group can be carried out with unabsorbed sera

Sh sonnei is essentially antigenically homogeneous although the regular occurrence of a phase variation characterized by the formation of smooth and rough forms of the organism gives rise to some confusion Both the S and the R forms are characterized by the possession of serologically distinct antigens which can be demonstrated by means of cross absorption tests In usual laboratory practice typing sera are employed which contain antibodies against both antigens

The chemical nature of the somatic antigens of the shigella group has been studied most fully in *Sh dysenteriae* by W J T Morgan *et al* (1949) and in various members of the *Sh flexneri* group by Goebel and his co workers (1945) and by Cluff (1954) The former used diethyleneglycol for the extraction of the organisms while the latter

two employed aqueous pyridine Although the toxic and antigenic properties of the various components isolated varied with the method of extraction the fractions themselves were of the same general nature The somatic antigen appeared to consist of a protein polysaccharide phospholipid complex thus resembling the classic Boivin antigen In general the carbohydrate was present in the form of a nontoxic polysaccharide which acted as a haptene to confer serologic specificity on the smooth organism this material was not found in the rough variant The lipid fraction was nontoxic and nonantigenic The protein fraction was represented by a nonantigenic-conjugated protein, which in the case of *Sh dysenteriae* 1 was identical with that present in *S typhi* The polysaccharide conjugated protein complex derived from the former organism is highly antigenic and apparently represents the simplest unit which will act as a complete antigen In their work with *Sh flexneri* 3 Tal and Goebel (1950) have presented evidence suggesting that the toxic factor of the somatic antigen is an entity distinct from the known protein lipid and carbohydrate components of the antigen complex

Toxins

The somatic antigens of the shigella as noted are chemically similar to those isolated from salmonella and coliform organisms and exhibit the same pharmacologic and physiologic properties as these analogous antigens of the other enteric bacilli In fact as discussed earlier the development of tolerance to any one of these endotoxins in man or animals by repeated injections renders the subject resistant to any endotoxin whether derived from an escherichia a salmonella or a shigella organism When shigella or other endotoxins are injected into rabbits or guinea pigs they cause diarrhea weight loss and inflammation and necrosis of the intestine These pathologic lesions closely resemble those found in clinical dysentery in man and it would seem reasonable to suppose that the symptoms and the pathologic changes seen in the natural disease in man are due in part at least to the release of endotoxin from the organisms in the gut However this theory fails to account for the fact that coliform and other enteric bacilli possessing similar endotoxins are present *in the intestine* in large numbers without producing similar effects This would appear to indicate that some other local effect is produced by shigella bacilli which allows their toxic components to penetrate the surface tissues where they can exert their effects *Sh flexneri* 2a synthesizes a mucinolytic enzyme which has been suggested as a possible mechanism for local penetration of organisms and toxic products (Formal and Lowenthal 1956)

In addition to these heat stable endotoxins which are identical with the somatic antigens *Sh dysenteriae* type 1 but not other shigella organisms produces another toxin Both smooth and rough forms of this organism elaborate a specific thermolabile toxin which is fully antigenic in and highly toxic for mice rabbits and guinea pigs Since this material may be separated easily from cell bodies it is usually referred to as Shiga exotoxin although there is no evidence that it is actually excreted by the living bacterial cells In general methods favoring the autolysis of cells give the highest yield Several methods for rapid production of this toxin have been described (Dubos and Geiger 1946) as well as the role of iron and related metals (van Heyningen 1955) and of carbohydrate metabolism and pH (Engley 1952) on the production of this substance It is protein in nature of the approximate molecular weight of 75 000 and relatively heat labile Injected into rabbits mice or guinea pigs it causes paralysis of the limbs diarrhea and death and because of the neurologic symptoms is sometimes referred to as the Shiga neurotoxin It is highly antigenic elicits the production of an antitoxin and may be converted to an antigenic toxoid by formalin (Farrell and Ferguson 1943) ultraviolet radiation (Branham and Habel 1946) and other means (Engley 1952) This toxoid produced local and systemic reactions similar to those caused by typhoid vaccine (Farrell 1944) in human volunteers In spite of its potency and profound toxic properties the possible role of this toxin in the pathogenesis of dysentery in man caused by *Sh dysenteriae* type 1 is unknown There is no convincing evidence that other species of shigella produce similar toxins

Variation

Arkwright (1921) described smooth and rough forms of *Sh dysenteriae* The observations have been confirmed and extended to other members of the genus by Waaler (1935) and Boyd (1938) The S to R variation resembles closely that described for other gram negative organisms It is associated with a change in colony formation and with the loss of the so-called endotoxin the toxic factor associated with the somatic antigen At the same time the type specific antigen disappears partially or completely Strains isolated from human cases of clinical dysentery are usually in the smooth phase The ability of rough forms to cause clinical disease remains undetermined

Among shigella the occurrence of phase 1 and phase 2 forms of *Sh sonnei* corresponding to smooth and rough forms has been described already Wheeler and Mickle (1945) felt that both these variants are essentially smooth in character although they might be distinguished by their characteristic colonial morphology and antigenic analysis However Kauffmann (1951) feels that this variation is a true S to R one In addition old laboratory strains frequently show a 3rd colonial variant which may be regarded as an extreme R form Boyd (1938) has described an unusual type group variation among strains of *Sh flexneri* Two types of colonies were noted on subculture of a given strain The 1st contained a type specific and a group specific antigen while the 2nd contained only the latter although it did give rise to a colony which still retained some of the characteristics associated with smooth strains Once the type specific antigen was lost there was no tendency to regain it Freshly isolated strains usually contained both the type specific and the group specific antigens

Shigella organisms exhibit antigenic variations which are related to the presence of specific temperate bacteriophages lysogenic conversion can be effected in different strains of *Sh flexneri* and this alters their antigenic identity (Iseki and Hamano 1959)

Bacteriophage

The gram negative bacteria inhabiting the gastrointestinal tract of man are usually susceptible to bacteriophage action as was first shown by d Herelle and in keeping with this specific phages effective against most members of the genus *Shigella* have been isolated In the case of *Sh flexneri* Burnet and McKie (1930) have shown that the various serologic types of this organism exhibit characteristic differences in their phage sensitivity Antigenically similar strains show practically identical reactions toward the series of phages tested Similar observations have been made in the case of *Sh sonnei* by Miller and Goebel (1949) and by Hammarstrom (1949) who was able to divide 1 834 strains of this organism into 68 types and subtypes by means of bacteriophage susceptibility In spite of its potential value for epidemiologic studies bacteriophage typing has yet to prove its usefulness in the identification of types and strains of dysentery bacilli

Natural Habitat and Range of Pathogenicity

The natural habitat of the shigella is the gut of higher primates notably man Other mammals such as dogs have rarely been found to be infected The naturally occurring disease is limited to man and perhaps apes and monkeys kept in captivity and these are the only species that develop recognizable clinical manifestations following the oral introduction of the specific organisms Of course contamination of food and water by human fecal discharges derived from carriers of these organisms may lead to the isolation of dysentery bacilli from these sources

Classification

As is common with most genera making up the family *Enterobacteriaceae* the classification of the shigella is neither easy nor entirely satisfactory The difficulties caused by the different nomenclatures used by American British and Continental workers is illustrated and discussed in the monograph by Kauffmann (1954) The most practical criteria of classification at present appear to be a combination of biochemical and antigenic characteristics Assuming the relative stability of serologic and biochemical characteristics of a given organism a useful classification is that proposed by the Shigella

Commission of the International Enterobacteriaceae Sub Committee (1954) The following classification follows the compromise scheme proposed by Cowan (1956) based on the report of this Sub Committee *Sh alkalescens* and *Sh dispar* are discussed briefly in a separate section at the end of this chapter since their membership in the genus *Shigella* is questioned by most authorities

CLASSIFICATION OF SHIGELLA

SCIENTIFIC NAME		COMMON NAME
Sh dysenteriae	Type 1	Shiga s bacillus
	Type 2	Schmitz s bacillus
	Types 3 7	Large Sachs bacilli
Sh flexneri	Types 1 6 plus 2 variants	Flexner s bacilli
Sh boydii	Types 1 11	Boyd s bacilli
Sh sonnei		Sonne s bacillus

PATHOGENESIS

Dysentery bacilli usually reach the gastrointestinal tract via the oral portal of entry through the medium of infected fingers food or water In direct contrast with the enteric fevers no bacteremic phase occurs and the organisms remain limited to the gut wall The essential pathologic process is an inflammatory one which always involves the large bowel and occasionally the terminal ileum as well Inflammation of the mucous membrane of the bowel wall is followed by necrosis which in more severe cases goes on to actual ulceration penetrating as deeply as the muscularis mucosa In contrast with amebic dysentery, the edges of the ulcers remain sharp and undermining does not occur In all but mild cases some degree of hemorrhage takes place The intervening mucosa is inflamed and edematous and microscopic examination of the bowel wall shows it to be infiltrated with polymorphonuclear cells As the process subsides granulation tissue replaces the ulcerative lesions and in severe cases scar tissue may develop

It must be admitted that the mechanisms responsible for these pathologic changes have not been clarified It is believed by some that the pathologic changes follow the local irritative action of the heat stable endotoxin released by the autolysis of the bacterial cells which are found in large numbers on the floor of the ulcers and frequently in the inflamed mucosa as well Direct experimental proof of this is lacking although the parenteral injection of large amounts of dysentery bacilli autolysates into laboratory animals gives rise to somewhat similar lesions Furthermore there is no clear-cut evidence bearing on what factors govern the severity of a given infection which may run all the way from an inapparent affair to a severe, rapidly fatal attack of dysentery

The classic clinical picture of bacillary dysentery is dominated by diarrhea abdominal pain and fever The incubation period is variable but may be as short as 24 hours Abdominal discomfort and cramps (often described as griping) are the first symptoms and usually come on suddenly They are followed shortly by diarrhea, which in all but the milder cases is accompanied by straining and tenesmus The stools are liquid almost from the start large amounts of mucus are passed and in the more severe cases blood as well The diarrhea and the abdominal cramps reflect the acute inflammation of the large bowel straining and tenesmus furnish evidence that the process involves the rectum The fever which accompanies the typical severe case presumably is due to the absorption of toxic products from the gut and to dehydration The disease tends to be self limited and uncomplicated recovery is the general rule Cases of chronic relapsing dysentery are usually of amebic origin Complications are rare A small proportion of recovered patients become chronic carriers of dysentery bacilli for varying periods of time These and even more so the individuals with inapparent infections constitute a major problem in the control of the disease

The clinical severity of a given case of bacillary dysentery is modified by nonspecific factors such as the age and the general condition of the patient and the opportunities for supportive therapy Some correlation exists between the species involved and the severity of clinical manifestations inasmuch as *Sh dysenteriae* 1 in general causes a more serious form of the disease than do other types or species

Immunity

The mechanism of spontaneous recovery from bacillary dysentery is not understood and attempts to elucidate the role played by various immune mechanisms have been much handicapped by the absence of a suitable laboratory animal in which a gastroenteritis may be established by the natural route of infection Although humoral antibodies ap pear in response to infection there is no evidence that they affect the recovery process directly nor is there a correlation between their presence or absence and the occurrence of relapses and second attacks Coproanti body has been detected in the intestinal dis charges of individuals suffering from bacillary dysentery (Barksdale and Ghoda 1951) and it is possible that this type of antibody may have a more significant relationship with the mechanisms of recovery and immunity in this disease The local tissue response may be a factor in this process the exudate in bacillary dysentery is rich in polymorphonu clear leukocytes and it is plausible that the presence of coproantibodies against the somatic antigen may enhance the opsoniza tion and subsequent phagocytosis of the in fecting organisms The use of the indirect bacterial hemaegglutination or hemolysis technics for the demonstration of small amounts of antibody may be the means whereby the role of these antibodies can be determined (Neter 1956) As in other enteric infections there is no evidence that bacteriophage plays a decisive role

Although relapses and second attacks of bacillary dysentery do occur it is common experience in the tropics that individuals set tled in areas where the disease is endemic tend to become immune to frank clinical attacks The nature of this clinical immunity is not clear and careful bacteriologic and immunologic studies will be necessary to de termine if it is species specific only or per haps broader giving some protection against other members of the genus as well

Although the injection of killed dysentery bacilli into human or animal subjects stimu lates the production of antibodies which con fer considerable protection on mice against the intraperitoneal inoculation of the homol ogous organism the results of controlled ex periments in which human volunteers were first vaccinated and then challenged by the oral route with the same type have shown no evidence of significant protection against infection (Shaugnessy *et al* 1946) Field trials (Hardy *et al* 1948 Higgins *et al* 1955a) have yielded similar results Inas much as the disease process is limited to the intestinal mucosa and there is no tendency to invade the bloodstream one would not ex pect humoral antibodies alone to be effective in the prevention of the disease Studies of the formation of coproantibody following im munization have yielded inconclusive results

Diagnosis

The laboratory diagnosis of bacillary dys entery is made most satisfactorily by the iso lation and the identification of the specific causative organism During the acute phase of the disease the organisms are excreted in large numbers in the feces and fresh stool cultures frequently give positive results par ticularly if the sample contains mucus as the bacteria are found in greatest numbers in this exudate (Boyd 1940) Since dysentery ba cilli tend to die out rapidly the stool must be cultured as soon as possible after being voided The suspension of fecal material in a preservative such as buffered glycerol saline solution is of some use if delay is unavoid able The rectal swab technic as first em ployed by Hardy *et al* (1942) offers prac tical advantages in the collection of specimens and their prompt plating on suitable media at the bedside but has the disadvantage of not permitting the microscopic examination of the stool for ova parasites and type of exudate During the acute phase of the dis ease there is little choice between this method and that of examining freshly passed stools as regards the percentage of positive iso lations Sigmoidoscopy with culturing of the actual ulcerations of the intestinal wall under direct observation (Ferris and Fortune 1944) yields the best results and is the method of choice in chronic stages of the disease when the organisms are relatively sparse

Mucus or failing this fecal material is emulsified in saline before streaking At the same time the type of cellular exudate may

be determined by examination of a simple stained smear A predominance of polymorphonuclear cells suggests dysentery of bacterial origin while a predominance of mononuclear cells favors a colitis due to *Entamoeba histolytica* Swabs are plated out directly As in the case of other enteric infections selective media are of the greatest advantage in primary isolation work

All these contain lactose and an indicator such as neutral red which will detect any fermentation of this sugar Such media permit rapid differentiation between prompt lactose fermenting organisms such as the coliform bacilli and late or non lactose fermenting bacteria such as the dysentery bacilli It is best to use 2 media one relatively noninhibitory such as MacConkey s agar and the other an inhibitory one such as shigella-salmonella-thiosulfate-citrate bile agar which largely suppresses the growth of coliform bacilli as well as that of gram positive organisms Selenite broth and other enrichment media are of limited usefulness in the isolation of shigella Suspicious colonies are fished and inoculated into Kligler s iron agar Those cultures showing an alkaline *slant and acid butt without the formation of* hydrogen sulfide or other gas are inoculated into carbohydrate broths (lactose glucose mannitol xylose sucrose salicin and dulcitol) for the detection of characteristic fermentation reactions into peptone water for the determination of indole formation into semisolid agar for the determination of *motility and on urea agar for the detection* of urease activity In connection with fermentation reactions it must be remembered that *Sh sonnei* is a late lactose fermenter which rarely attacks this sugar before 48 hours and usually later

Once an organism has been grouped provisionally by means of these biochemical reactions its final identification should be carried out by the use of specific typing antisera employing the method of slide agglutination

Since as was first shown by Shiga in 1896 agglutinins appear in the bloodstream during the course of the disease it is theoretically possible to make a serologic diagnosis by the demonstration of the formation of specific antibodies against a given organism during the course of illness At best this furnishes a retrospective diagnosis and in actual fact this technic is relatively unsatisfactory, since *these antibodies develop irregularly*, and the multiplicity of species and types capable of causing the disease necessitates the setting up of the agglutination test against a large *number of bacterial suspensions*

Specific Treatment

Antimicrobial therapy with the broad spectrum drugs *(tetracyclines and chloram*phenicol) has been markedly effective in the treatment of bacillary dysentery As the result of extensive field trials carried out in the prisoner-of war camps in Korea Hardy and his associates (1952, 1963) demonstrated that the administration of any of these drugs in relatively small amounts over a period as short as 24 hours resulted in the prompt disappearance of the clinical manifestations in nearly all cases This clinical improvement was paralleled during the same time period by a rapid conversion from positive to negative cultures As yet the emergence of drug resistant organisms and the occurrence of relapses have not constituted significant problems This stands in *marked contrast with earlier experience* with sulfonamide drugs During World War II many of these agents were shown to exert a bacteriostatic effect on members of the genus *Shigella in vitro* and at first clinical trials with both the soluble compounds (e g sulfadiazine) and the insoluble ones (e g sulfaguanidine) yielded markedly favorable *results However the rapid* emergence of drug resistant strains (particularly *Sh flexneri* 3 and *Sh sonnei*) has sharply limited the usefulness of these agents and today the broad spectrum antibiotics mentioned above are the drugs of choice

Serotherapy has been disappointing and today finds little use Convincing proof of the efficacy of high titer antitoxin against the so-called exotoxin of *Sh dysenteriae* 1 in modifying the course of disease caused by this organism is lacking A polyvalent antiserum has been used in the treatment of infections due to the various types of *Sh flexneri* with little or no success As in the case with other enteric infections the results obtained with bacteriophage have been disappointing The carefully controlled experi

ments which Boyd and Portnoy (1944) carried out in North Africa during World War II may be cited as an example of a field trial in which no evidence of therapeutic or prophylactic efficacy on the part of the bacteriophage preparations employed was obtained

Epidemiology

Bacillary dysentery is traditionally regarded as a disease of the tropics however in actual fact it has a world wide distribution *Sh flexneri* and *Sh sonnei* present a particularly wide distribution *Sh dysenteriae* 1 usually associated with a clinically severe form of the disease has been found in significant numbers only in the Far East in recent years and even here its importance as a causative organism of the disease appears to be on the wane *Sh sonnei* is encountered most frequently in the United States

The source of infection is essentially man The shigella are strict parasites and no natural animal reservoirs have been detected Their natural habitat is the gastrointestinal tract of man Here they may cause a fulminating case of classic dysentery a mild diarrhea or perhaps most frequently, an inapparent or subclinical infection Because of this wide variation in severity of clinical symptoms the term shigellosis is often applied to infections (apparent and inapparent) caused by this group of organisms It is well recognized that the organisms are excreted in large numbers during the acute phase of clinical dysentery It is not so well recognized that the patient with a mild diarrhea or with no symptoms whatsoever may also be acting as an excretor of the organism As the patient recovers from the clinical attack of the disease the organisms tend to disappear concomitantly from the stools although in some instances they may persist during convalescence or even longer The development of a true carrier state is of rare occurrence perhaps due to the fact that dysentery bacilli show no tendency to invade the biliary tract Clinical attack rates are higher in children as compared with adults in the same community Presumably the development of a specific immunity plays some part in this finding

The spread from man to man may take place by a variety of methods The contamination of human fingers with fecal material containing dysentery bacilli is the first step in the direct transfer of organisms from one individual to another (Hutchinson 1956) The contamination of inanimate objects such as toilet seats door handles toys pottery and glassware which are used in common by many people represents an intermediate step in the process Outbreaks occuring in institutions such as asylums or enclosed military groups are usually due to this type of spread as a general rule as standards of personal hygiene improve the incidence of shigella infections diminishes As Watt (1956) has emphasized a high prevalence of these infections in a given community usually is associated with the more or less direct transfer of infected human feces from one individual to another The effect of water availability on the prevalence of shigella infections has been studied by Hollister (1955) and others In general infection rates were found to be highest in areas where water was least available for personal hygiene

Spread from man to man may occur more indirectly through the medium of contaminated food or water supplies Foodstuffs are usually infected by the dirty fingers of food handlers or by flies which have access to fecal material containing dysentery bacilli Genuine water borne outbreaks have been comparatively rare and usually occur through the medium of faulty plumbing or lack of protection of the source of supply In warm areas where there is usually a large fly population these insects may play a major role in the spread of these organisms if they are permitted to feed indiscriminately on human feces and food It should be emphasized that the transmission of shigella by these insects is primarily a mechanical one as there is little evidence that multiplication takes place to any extent in the gut of the fly The low standards of environmental sanitation frequently found in the tropics and the presence of a large fly population are important factors in the high prevalence of clinical dysentery in these areas

As noted earlier bacillary dysentery still occurs in areas with high standards of sanitation and outbreaks caused by *Sh sonnei*

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formerly classified with the shigella When studied in greater detail both biochemical and antigenic properties reveal that they are coliform organisms (Stuart *et al* 1943) Though they have been isolated from patients with dysentery their etiologic role in this disease is questionable but like other coliform organisms they do cause urinary tract infections

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are seen in which the family unit may maintain the infection with subclinical infection for weeks with various members and contacts developing clinical disease at intervals (Neter 1962)

Bacillary dysentery spreads easily within the hospital unless careful precautions are taken outbreaks of *Sh sonnei* infections are frequently reported

Control Measures

The logical control of bacillary dysentery falls under 4 headings (1) elimination of sources of infection (2) prevention of spread (3) increasing individual resistance and (4) chemoprophylaxis Of the 4 the second i e prevention of spread has played through the application of a variety of community and individual measures the dominant role in reducing the prevalence of the disease Since man represents the only recognized host of the shigella the elimination of these organisms from man should remove the great reservoir of the disease At present this is an unattainable goal since the detection and the isolation of clinically mild and of inapparent infections still present insuperable problems As pointed out previously, inapparent infections probably outnumber clinical cases of the disease by a considerable margin These facts limit the extent to which the elimination of the source of infection can be carried (Philbrook *et al* 1948) The intermittent shedding of organisms by convalescent patients and healthy carriers presents a further complication Nonetheless rigid precautions should be taken with the excreta of patients suffering from recognized infections and these individuals should not be released from isolation until stool or rectal swab cultures are negative on 3 or more successive days

The spread of organisms from one infected individual to another can be reduced by measures falling into 2 large groups On the individual basis high standards of personal hygiene will do much to prevent direct spread from one individual to another The so-called asylum dysentery found both in the United States and Western Europe is a case in point Bacillary dysentery is often endemic in large mental asylums and in orphanages where overcrowding and poor sanitary habits of mentally defective youthful inmates gives every opportunity for the transfer of fecal material from one individual to another The thorough washing of hands, particularly after defecation and before meals, is one of the simplest and most effective methods in reducing this type of spread On a community basis those sanitary measures aimed at providing a safe supply of water milk and food to the community are all important, as is the operation of an adequate sewage disposal system As has been demonstrated by Watt and Lindsay (1948) effective fly control is often a potent factor in reducing the prevalence of the disease in communities where both food and human excrement are not protected from these insects

Attempts to increase the resistance of the individual by active immunization have not been successful to date The toxicity of available vaccine preparations and the multiplicity of potential etiologic agents are factors which have handicapped trials of this method As yet no significant relationship has been proved between protection against the clinical disease and the presence of humoral or coproantibodies

The efficacy of certain antimicrobial agents in eradicating infections with dysentery bacilli has given hope that their use in chemoprophylaxis may exert a significant effect on the prevalence of disease During World War II mass chemoprophylaxis with relatively small amounts of sulfadiazine was reported to be effective in checking outbreaks of the disease However the emergence of drug resistant strains soon made this type of chemoprophylaxis ineffective The demonstration of the effectiveness of the tetracycline drugs in the treatment of these infections renders it logical to employ these agents in chemoprophylaxis as well Although the reported experience of Higgins *et al* (1955b) in Egypt was not encouraging further field trials under controlled conditions are indicated in order that the potential efficacy of this means of prophylaxis may be evaluated

THE ALKALESCENS DISPAR ORGANISMS

These organisms which are nonmotile fail to ferment lactose do not form gas from other carbohydrates and behave like shigella organisms in carbohydrate reactions were

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26

The Vibrios--Cholera

Vibrios are polymorphic gram negative bacteria which under optimal conditions of cultivation appear in fixed smears as curved rods Most strains are highly motile and are facultative aerobes

The word *vibrio* is used in both a mor phologic and a generic sense With the ex ception of a few highly specialized species all vibrios are at present included in the genus *Vibrio*

Vibrios comprise a vast subcontinent of the microbial world a region only super ficially explored Attention has been so con centrated on *Vibrio cholerae* that the dimen sions of the genus are frequently overlooked It includes strains isolated from serious in fections in mammals birds fish reptiles amphibians and insects Vibrios are readily found in water soil and decaying vegetable matter There are halophiles thermophiles pigment producers aerobes and anaerobes Some strains have highly specific substrate requirements but many adapt themselves to a wide variety of substrates with the greatest facility It is somewhat paradoxical that al though the generic classification is morpho logic many vibrios will assume morphology varying through a wide range depending on environment The genus *Vibrio* is not only complex it is often regarded as chaotic Be case of the enormity of the field no com pletely acceptable taxonomic system has been achieved

MORPHOLOGY

Under optimum conditions of cultivation vibrios appear as curved rods 1 to 5 microns long and 0 25 to 0 35 micron broad Usually only about half of the organisms in a given smear appear curved this being a matter of position on fixation. The typical shape is that of the German comma In any prepara tion there may be a considerable variation of morphology and each strain is broadly characterized in this sense Thus some show many S shaped forms whereas others show only stubby slightly curved forms

All vibrios autolyse readily Under most conditions the disintegration gives rise to small dense granules These are believed to be reproductive units (in part at least) and account for the fact that old style porcelain filters do not give sterile filtrates with vibrios It is sometimes possible to use this fact for separation of vibrios from other species

Under conditions not too favorable for multiplication (as with small amounts of penicillin) vibrios may evolve as structures quite long and without curvature These have been referred to as mycoidal If large amounts of penicillin are employed, the cell walls are lost and the organisms appear as round bodies of various sizes Although spheroidal these forms maintain directional motility

Long spirillar forms were demonstrated by Koch in the rice water stools of cholera patients Although these forms can be demon strated readily under such circumstances they have not as yet been produced in vitro The term fish in-stream was used by some workers to describe their findings in cholera stools but it is probable that these represented early autolysis of the spirillar form due to delay in making the smears

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Certain precautions are necessary since some vibrios utilize nitrites as well as nitrates Hence if a negative result is obtained a test with zinc dust should be done to demonstrate that nitrate remains A second precaution is that the concentration of nitrate should not exceed 0 005 per cent if the same medium is to be used for indole detection Nitrites inhibit indole formation The cholera red test applies rather generally to organisms which form indole and reduce nitrates but it has no real value in classification

Carbohydrate fermentation is a most useful feature for purposes of general classification This system was devised by Heiberg and utilizes sucrose arabinose and mannose Although 8 combinations are possible only 6 are required to describe cultures in present collections

Group	I	II	III	IV	V	VI
Sucrose	A	A	A	A	—	—
Arabinose	—	—	A	A	—	—
Mannose	A	—	A	—	A	—

All fermentation cultures of this type are essentially competitive affairs Acid is formed from carbohydrates but alkaline products result from peptone utilization With many cultures there is something of a race the final pH being the result of the particular preferences involved or the relative amounts of substrates presented Not infrequently the surface of the broth will be alkaline and the butt acid Fermentation of mannose at least should be carried out under a layer of sterile paraffin oil

The use of these 2 schemes gives 48 combinations but is far from definitive of species These combinations are merely broad group descriptions and can be indicated as a I b IV etc

Some vibrios produce soluble hemolysins in broth cultures The original demonstration was with organisms grown in alkaline peptone water for 3 days at 37 C Such hemolysin is produced by the original El Tor strains and is quite stable A second type of hemolysin is found with many other organisms at 24 hours in low titer but usually disappears completely by the 3rd day

RESISTANCE

Vibrios in general are very sensitive to acid the usual chemical antiseptics and to many aniline dyes They are destroyed at 55° C in 15 minutes Drying is regarded as an adverse influence but some features are not easily explained In the laboratory cultures do not tolerate refrigeration well

VIBRIOPHAGES

Many lines of bacteriophage active against vibrios have been recovered from a variety of sources The possibilities of this approach to taxonomy and to epidemiology are almost unlimited Most of the findings relate to *V cholerae* and will be discussed under that heading

ANTIGENIC STRUCTURE

Flagellar antigens (H) are found in all motile vibrios The addition of anti H serum to suspensions of homologous vibrios gives a flaky or flocculent result A number of flagellar antigens have been postulated but this subject is not yet clarified The flagellar substances of vibrios do not appear to be highly antigenic

Somatic antigens (O) are quite dominant as antibody producers with all vibrios Burrows has clearly demonstrated that some strains of *V cholerae* possess as many a 6 differing somatic antigens He recognized a total of 14 such antigens in various strains of *V cholerae* With a single injection of living cholera vibrios animals give antibody response to not more than 3 antigens *V cholerae* generally gives response to 2 One of these (usually designated as A) is dominant and is found in all cholera vibrios With repeated injections over a period of time antibodies develop to other presumably minor antigens

An unfortunate terminology has come into use namely that vibrios other than the cholera vibrio are called nonagglutinable (NAG) The fact is that all smooth vibrios are agglutinable by the proper reagents It is more useful to use the phrase noncholera vibrios (NCV) Gardner and Ventkatramen pointed out that a serologic classification of

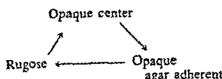
Vibrios are readily stained with aniline dyes and are gram negative

Most vibrios are motile They usually possess a single polar flagellum

GROWTH REQUIREMENTS AND CULTIVATION

Most vibrios can be grown on synthetic media containing asparagin and a suitable mixture of mineral salts They grow well on ordinary laboratory media except for some dye-containing media used in enteric work The optimum temperature for most vibrios is near 32° C but growth is possible between 10° and 39° C Anaerobic growth is usually rather slow The optimum pH for the enteric vibrios is near 7 5 but growth will occur with alkalinity as high as pH 9 5 On the other hand vibrios are rather sensitive to acid Growth usually cannot be initiated if the pH is 6 2 or lower In the presence of a fermentable carbohydrate vibrio cultures undergo autosterilization due to the acid formed

On any good peptone agar the colonies of vibrios are somewhat varied No general description applies since colonies may be clear semitransparent or opaque As the colony develops the term opaque-center is frequently applicable especially with the type species *V cholerae* On enriched media such as brain heart infusion agar any given culture is apt to display some variation quite characteristic of that strain Two such types of variation are found in cultures of *V cholerae* The first is the opaque⟷transparent colony system This is usually associated with nonpelliculate strains The second is



This is frequently found with pellicle forming strains

Although a wide variety of pigments have been observed with vibrios those isolated from mammalian infections are usually regarded as non pigment formers Actually there is a small amount of tan to brownish pigment but this gives little contrast to the color of the medium save in older cultures Some cultures give colonies with a slight iridescence in oblique light

Colonial morphology does not clearly reflect the S⟷R system of variation Isolates regarded as rough because of granular growth in broth and which lack the antigenic attributes of smooth forms usually give colonies with rather smooth surfaces

All smooth vibrio forms give an initial even turbidity in broth The rate of development of turbidity (not entirely related to the number of viable forms) is characteristic of any given strain and indeed this simple description often applies to all strains from a given cholera epidemic Some strains characteristically form a pellicle which adheres firmly to glass and has a tendency to climb The nature of the pellicle is not understood it does not appear to be simply a collection of oxygen loving organisms

One of the more sensitive areas of growth requirements is in the matter of salt Even those vibrios isolated from fresh water sources do not thrive unless furnished NaCl at levels of 0 5 to 1 0 per cent Levels of 10 per cent are often required for isolates from sea water

BIOCHEMICAL ACTIVITIES

Most vibrios are far from fastidious and will tend to adapt to available nutrients This requires standardization of approach if reproducible results are to be obtained

The earliest biochemical work had to do with the separation of cholera vibrios from all others (then called water vibrios) This involved the liquefaction of gelatin the production of indole and the reduction of nitrates to nitrites This system still serves as an excellent beginning toward division of the vibrios into large groups Thus

GROUP	a	b	c	d	e	f	g	h
Gelatinase	+	+	+	+	−	−	−	−
Indole	+	+	−	−	−	+	+	−
Nitrite	+	−	−	+	+	+	−	−

All of these combinations exist All cholera vibrios belong to Group a

been used to improve circulation but these are *probably quite ineffective during the* period of collapse Unfortunately the proper management of therapy is not always feasible in the rather remote regions where the disease is most disastrous

Etiology It is usually stated that Asiatic cholera is a specific disease entity in which the etiologic agent is *V cholerae* This statement can be debated at almost every point It is very difficult to give more than a broad definition to the disease entity for the usual description applies only to those patients who are seriously ill No one can be quite certain of the extent of mild cholera a disease state resembling diarrheas due to many causes The position of *V cholerae* as an etiologic agent *per se* is even more tenuous Although *V cholerae* is regularly isolated from feces of these severe diarrheas it has never been possible to obtain proof of etiology since an identical disease entity has not been regularly reproduced in man or animals Indeed the failure to develop cholera following laboratory misadventure and the lack of secondary cases in hospitals have often been cited as evidence against the idea of specific etiology The specific etiologic hypothesis rests largely on the fact that specific antibodies against *V cholerae* develop in the course of convalescence

Although *V cholerae* is isolated from feces of every severely ill patient other vibrios have been isolated (1) from the same individuals late in the disease and (2) from milder choleralike states frequently as the only bacterial form present These isolates of noncholera vibrios represent about 75 immunologic types and include strains in biochemical categories aII aI aV dIII eII and aIV The relationship of the noncholera vibrios to cholera vibrios can be the subject of much speculation It is altogether possible that some sort of phase variation is fundamental to the cholera problem Noncholera vibrios have been isolated from well established clones of cholera vibrios and clearly identified The reverse is also true but the factors or forces operative are completely unknown

The actual process of pathogenesis of cholera is unknown It is assumed that the vibrios are acquired in water or food and that they somehow pass safely through the adverse acid environment of the stomach and then multiply enormously and bring on diarrhea Much of this hypothesis presents difficulty for vibrios are extraordinarily sensitive to acid they generally multiply rather slowly under the reducing conditions prevalent in the gut and they do not thrive well in the face of competitive intestinal flora However the initial diarrheal samples are loaded with vibrios the numbers tend to diminish rather rapidly Rice water stool itself constitutes a very poor culture medium

There is no evidence of parenteral invasion during the disease Vibrios have been isolated from various organs and tissues postmortem but positive blood cultures are unknown However the vibrios or their products must find their way to antibody producing cells for convalescent antibody titers can be quite high

The species *V cholerae* includes a considerable variety of microorganisms All strains have the biochemical formula of a I that is all liquefy gelatin form indol reduce nitrates ferment sucrose and mannose but not arabinose All possess a common group somatic antigen (A) All possess in addition a second strong somatic antigen either Ogawa (B) or Inaba (C) There are descriptions of organisms having the somatic formula ABC (Hikojima) but there is confusion as to this point Although the foregoing may be taken as the present species definition the description includes a large group of vibrio lines These lines have meaning in epidemiology and may have meaning with reference to severity of infection Some examples of possible subclassification may be offered

A *Morphology* Some strains differ markedly from others but a firm classification is not yet possible Nonmotile strains of *V cholerae* have been isolated from feces but the significance is not known

B *Immunologic Characteristics* The subtypes Ogawa and Inaba are readily identified with properly absorbed antisera It is usual that any outbreak is characterized by the dominance of one type However it is not unusual that the other will make its appearance late in any series In stock cultures subtypes are quite stable

C *Biochemical Characteristics* All strains ferment dextrin galactose glucose levulose

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As applied to the total taxonomic problem it should be pointed out that the three systems of classification (basic biochemical, carbohydrate fermentation and immunologic) are quite independent Even definition with all three systems is not necessarily sufficient for the definition of a species It is probable that this 3 fold description plus description of special features of irregular nature should form an adequate species characterization at this time

INFECTIONS ASSOCIATED WITH VIBRIOS

As understood at present, vibrios may be associated with human disease states in three categories (1) the enteric vibrioses, (2) infectious states derived indirectly from vibriotic epizootic diseases, and (3) opportunistic occurrence in various disease states

The Enteric Vibrioses (Asiatic Cholera)

Asiatic cholera is an acute infectious disease characterized by abrupt onset vomiting extraordinary diarrhea muscular cramps and other signs of dehydration subnormal temperatures, fall of blood pressure suppression of urine, and rapid collapse It is associated with the presence of vibrios in the rice water stools these usually being identified as *V cholerae*

History Cholera has been endemic in the delta of the Ganges and the Brahmaputra (India and East Pakistan) since remote times During the 19th century there were repeated pandemics involving most of the world The United States was invaded in 5 of these waves but has not experienced the disease since 1873 From the chief endemic focus in Bengal (and perhaps a minor focus in China) the disease has shown repeated extensions At the present time cholera appears to be well seeded in India Pakistan China Malaysia the East Indies, the Philippines etc Its present northern reach is Korea Its easterly march has come to New Guinea A large proportion of the world s population is at risk of exposure

Clinical Course The incubation period is not clearly established but probably varies from a matter of hours up to 5 days There are usually no clinical events preceding the abrupt vomiting and diarrhea The loss of fluid is acute often a matter of liters within the first 5 hours There are cramps of great severity in the legs and the feet The patient sinks into a condition of collapse the features are sunken, the skin is wrinkled and clammy the temperature falls, the blood pressure falls, the pulse is extremely feeble and flickering and the patient becomes stuporous The first feces are yellowish but quickly become watery with whitish particles (rice water) With recovery there is first the appearance of biliverdin in the stools and then with the re-establishment of the normal flora the familiar brown color reappears The symptomatology corresponds in degree to the amount of fluid lost in the feces During the worst of the collapse urine flow is slight and may cease altogether Death may occur during the period of initial collapse or at a somewhat later time due to failure of kidney function The death rate in untreated cholera has sometimes been quoted as high as 40 per cent but this figure is relative to the number of severe cases and without great meaning Recovery often occurs quite rapidly and there appears to be no residium

Cholera shows no characteristic postmortem pathology aside from evidences of dehydration Older descriptions of changes in the mucosa of the lower ileum and the ascending colon are now recognized as related to postmortem events Physiologic studies in cholera have demonstrated the enormous fecal losses of water and of bicarbonate sodium potassium and chloride ions Therapy is based on orderly replacement of these losses Since the diarrheal loss is primarily isotonic the physiologic replacement is primarily isotonic saline by the intravenous route Acidosis may need corrective attention after initial rehydration has been accomplished The use of broad spectrum antibiotics such as tetracycline has been advocated These may perhaps shorten the period of diarrhea Many drugs have

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tive transport of sodium The blocking agent has not been precisely identified but it behaves as an anion of low molecular weight (perhaps a few hundred) which is stable to heat and to weak acids and alkalis It is not removed with liquid solvents This blocking agent has been demonstrated in culture filtrates and in fecal samples from cholera patients It has probably been present as a contaminant of various preparations of toxins mucinases etc

If this inhibitor exists in free form it is most unlikely that it would be antigenic perhaps it actually exists as the effector grouping of a larger molecule

The toxin theory of cholera has a long history The subject is confused by the number of toxins isolated and by the fact that some of these have been isolated from non cholera vibrios The confusion in this problem has been well reviewed by Jenkin and Rowley The relationships between the various toxins remains obscure Their toxic effect in mice is never accompanied by diarrhea The participation of toxins in the disease is thus uncertain

The only experimental infection with reasonable resemblance to cholera is the infection of infant rabbits as developed by Dutta and Habbu (1955) After gastric lavage rabbits 8 to 10 days old are starved for 18 hours After a second lavage the animal is fed with culture This is followed regularly by diarrhea in 6 to 30 hours The animals lose more than 10 per cent of body weight the skin is cold the hair rough and the underside of the body smeared with liquid feces Death occurs in 10 to 48 hours At death the large intestine is full of fluid and the small intestine shows a moderate degree of congestion Liver spleen and kidneys are generally normal This result can be produced with cultures with culture filtrates and with cholera fecal filtrates It was originally ascribed to an endotoxin Unfortunately this experimental infection is not suitable for immunologic exploration

There have been many experiments in which cultures or culture filtrates introduced into isolated intestinal loops have produced an inflow of fluid so that the loops become distended These results have been variously interpreted as related to mucinase or to endotoxin

IMMUNOLOGIC FEATURES

The finding of strong antibody response with cholera casts some doubt on the usual theory that there is no invasive process in this disease By the 5th to the 7th day there is a sharp rise of specific serum antibody titers This does not correspond to the time of recovery It has been shown that a number of patients on admission have serum antibody directed against the group somatic antigen (A) but none has shown antibodies against the type antigens (B C) In convalescence most patients show marked antibody rise against both group and type antigens

It should be pointed out that serum antibody does not necessarily correspond to coproantibody The latter may indeed appear and exert effects earlier than the serum antibody Upon artificial immunization coproantibody diminishes much more rapidly than serum antibody

There is no convincing evidence of acquisition of permanent immunity on recovery from an attack of cholera Much that is advanced is untenable because it involves clinical diagnosis a matter of much difficulty since during epidemics almost any severe diarrhea is called cholera

EPIDEMIOLOGY

Some epidemic situations in Europe in the 19th century were shown to be water borne It had been widely recognized that the disease spread from one community to another on a rather deliberate schedule and it was thought to be carried by persons or by fomites With the passage of time the matter of epidemiology has not been resolved especially in the endemic regions The interepidemic reservoir is unknown

In the Bengal region the disease shows two waves each year In the fall there are usually sharp outbreaks in various communities but the number of cases is comparatively low In the late spring there is usually a major wave in which many communities are struck

maltose, mannose, ribose sucrose and trehalose Negative results are obtained with adonitol arabinose cellobiose dulcitol erythritol inositol inulin melibiose raffinose rhamnose salacin sorbitol and xylose Most strains ferment mannitol but others are characteristically mannitol negative

D *Growth Characteristics in Broth* A sharp division between pelliculate and non pelliculate strains exists Growth patterns (based on turbidity) differ widely and appear to be rather constant characteristics of given strains

E *Hemolytic Activity* The original El Tor vibrios produced a stable hemolysin as determined in filtrates of 3 day broth cultures (37° C) These organisms were recovered from normal persons and were regarded as nonpathogenic In earliest usage the term El Tor was applied to all vibrios producing this stable lysin, whether cholera vibrios or not Later the term was restricted to cholera vibrios The term is now being applied also to cholera vibrios which give some hemolysin at 24 hours (None is usually found at 3 days) Although the associated disease is clearly cholera the terms El Tor enteritis or choleriform enteritis have come into vogue Some strains in these epidemics fail to produce any hemolysin These are designated as nonhemolytic El Tor Considerable confusion arises as a result of such evasive terminology

F *Bacteriophage Typing* As a result of study of a large number of phage lines against a long series of microorganisms Mukerjee has provided an important tool for the subclassification of *V cholerae* Four phage lines well defined are used for this classification At the present time while the total range of possibilities is under exploration it seems best to describe the actual results rather than to categorize with numbers Bacteriophage typing is probably one of the most sensitive tools available at this time but it requires expert handling

LABORATORY DIAGNOSIS

Smears of rice water stools generally reveal the spirillar forms described by Koch but much experience is required with this method since there is little staining contrast with background material

Many schemes have been developed for the cultural isolation of enteric vibrios Success is related to familiarity with the given method A simple and satisfactory system employs an agar medium containing 3 0 per cent gelatin 1 0 per cent trypticase 1 0 per cent sodium chloride and 0 1 per cent yeast extract After 15 to 18 hours enteric vibrios give colonies surrounded by sharp zones of clouding (Other proteolytic organisms give fainter zones of clouding by 48 hours) Serologic typing (by slide agglutination) can be made directly from these colonies Monsur has modified this medium by the addition of tellurite enteric vibrios give grey to black colonies with a halo

Without question the most satisfactory results are those obtained by immediate streaking of culture medium with feces or with material obtained by rectal swab The preservation of samples is never completely satisfactory but the preservation medium of Monsur is recommended

The isolation of *V cholerae* (or other vibrios) supports a clinical diagnosis of cholera For more definitive work it is preferable to demonstrate a 4 fold rise of antibody titer between acute and convalescent sera

The significance of noncholera vibrios cannot be assessed yet It is not unusual to find these organisms exclusively in fecal cultures At other times they are found along with *V cholerae*

PATHOGENICITY

The most probable explanation of the diarrhea of cholera is that *V cholerae* produces a substance of low molecular weight which inhibits or blocks sodium transport in the terminal ileum and the ascending colon This possibility has been demonstrated by the frog skin technic by Fuhrman and Fuhrman by Phillips and Huber and by Fuhrman Fuhrman and Burrows One scheme of thinking holds that large volumes of fluids are almost constantly introduced into the intestinal tract and that the resorption of this water is largely an accompaniment to ac

measures if they understand the reasons involved

OTHER VIBRIOSES OF MAN

There are a large number of vibriotic infections in domestic animals e g contagious abortion in cattle (*V fetus*) swine dysentery (*V coli*) and vibrionic diarrhea of calves (*V jejuni*) Many of these vibrios are microaerophilic grow very slowly and are difficult to characterize Most have been incriminated as infectious agents in man Usually direct contact has been involved The human infections have been varied presumably depending on the portal of entry The number of such infections is unknown since the organisms may not be recovered by routine procedures Antibiotic treatment is effective in these human infections

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sharply and all communities show some cases This wave usually terminates with the monsoon In other areas now involved in cholera the time schedule is less regular

It is not uncommon that two or more persons in a given household will develop cholera Usually others in the same household excrete *V cholerae* but do not develop the disease It is frequently impossible to trace these episodes to contact with the disease It must be surmised that either an isolated water source or food may have a significant role It is curious that the rates frequently rise sharply after any feast day (including Sundays)

All individuals convalescent from cholera show vibrios in feces for a variable period of time up to about 3 weeks There is no solid evidence to indicate that convalescent carriers are sources of new infections Individuals from households in which cholera occurs are frequently healthy carriers Inapparent carriers have not been incriminated as sources of other infections, but this seems to be a most likely possibility Permanent carriers are as yet unknown

In most of the cholera areas, noncholera vibrios exist in abundance but there is no evidence to support the conjecture that these undergo conversion into cholera vibrios

The epidemiology of cholera is thus completely unresolved In recent decades cholera has confined itself generally to individuals who exist under the lowest economic circumstances Yet in these regions there are many such who seem never to be caught Who then is the target individual? What constitutional or environmental circumstances put him in line for this tragic physiologic upheaval?

CONTROL OF CHOLERA

Immunization It is common practice to give an injection of cholera vaccine in the face of an epidemic Persons entering cholera areas usually receive at least two injections Although all observers have much faith in this approach there is no acceptable proof of the virtue of immunization against cholera However wide use of vaccine has been followed by a cessation of an epidemic so frequently that it must be accepted as the primary weapon In a broad sense, it is probably not too effective unless 80 per cent of the population can be brought into the program

Cholera vaccine is a suspension of killed vibrios in phenolized saline It should contain approximately 8 000 million vibrios per ml this being made up of equal quantities of Ogawa and Inaba strains Actually the vaccines are prepared on a turbidometric basis In the United States the vaccines are evaluated on the basis of a mouse protection test using a mucin system similar to the procedure employed with typhoid vaccine This method is quite impractical in many of the cholera areas where cholera vaccine is often prepared locally and is not evaluated There have been a number of misfortunes related to this matter since in a number of places there has not even been identification of the microorganisms employed The term cholera vaccine has covered many things This lack of international definition of quality and potency standards may well be the greatest stumbling block in any program directed toward definitive evaluation of cholera vaccine and its possible use in a worldwide control program

Sanitary Measures It is the custom to make every effort to provide sterile household water but this is almost impossible in some regions Despite health education procedures such as boiling of water proper disposal of excreta etc are not always accepted and practiced

Quarantine Measures Restrictions are usually imposed on the movement of people *and their goods and against shipment* of various foodstuffs These regulations are based on very old ideas and there is some question as to their effectiveness Perhaps in the day of movement by ship these points were valuable

In some recent outbreaks all persons of cholera households and all known contacts of cases have been put under observational arrest Then the proved carriers have received antibiotic treatment

Public Information The most successful programs of control in recent years have involved the enlistment of the public in all efforts A cholera epidemic is almost beyond the resources of any official group The people will support even the strongest police

measures if they understand the reasons involved

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Morphology

P multocida varies from small short oval forms to coccobacilli with convex sides and rounded ends Their length ranges from 0 3 to 1 25 microns, their diameters from 0 15 to 0 25 micron and they appear singly in pairs chains or clusters Healthy organisms from cultures stain easily and diffusely with aniline dyes and are gram negative in animal tissues and fluids the ends of the rod stain more intensely than the central portion does The bipolar staining corresponds to the location of the chromatin bodies or of reserve substances surrounding the chromatin granules *Pleomorphism is not common* but filamentous granular or barred bacilli without bipolar staining may be seen in preparations from rough colonies The organisms do not form spores and are not motile

Capsules when present can be shown in counterstained India ink or Giemsa-stained preparations one of the most useful methods is to make a smear in a loopful of 10 per cent serum and after drying and fixation stain with carbol fuchsin diluted 1 4 with water (method of Jasmin 1945 modified) bacteria and background stain red while the capsules remain colorless

Cultivation and Biochemical Activities

Nicotinamide pantothenic acid and aneurin are essential growth factors and cystine is apparently the only essential amino acid (Jordan, 1952b) Lactic acid or sucrose provides an adequate carbon source and improves capsule production Growth from small inocula is often inhibited by the presence of peroxides in the medium hence the usefulness of adding blood hematin catalase or sodium sulfite (Jordan 1952a) or of incubating anaerobically Practical media have been described by Namioka and Murata (1961) Colonies of smooth strains *reach 1 to 1 5 mm in diameter after* 24 hours at 37° C No hemolysis occurs but most strains discolor blood agar brown Cultures on solid media have a faint but distinctive smell Broth cultures of smooth or mucoid strains are evenly turbid rough strains produce a floccular or granular deposit The optimal growth temperature is 37° C but many strains also grow at 42° C Drying sunlight, heating above 50 C 0 5 per cent phenol and 0 1 per cent formalin are lethal in 15 minutes and cultures tend to die after a week or two on the bench or in the refrigerator

Four principal colonial variants are recognized (Carter and Bain 1960) Mucoid (M) strains produce the largest colonies reaching 2 to 3 mm in diameter after 24 hours and preponderate in freshly isolated *cultures of the serologic type* A They possess a thick capsule containing a hyaluronic acid and hyaluronidase produces decapsulation (Carter 1958) The mouse pathogenicity *of mucoid strains varies greatly* Smooth capsulated (S) variants produce smaller colonies and have a less marked capsule They are almost always highly mouse pathogenic and cultures on transparent *solid media* such as serum agar usually show iridescent colors including green and blue in obliquely transmitted light this is believed to be a diffraction effect due to capsular *material* (Smith 1958) Smooth noncapsulated (S^R) variants resemble these but capsule and colony iridescence are slight or absent Their mouse pathogenicity varies and *many strains* are highly pathogenic in the absence of any demonstrable capsule or iridescence Rough (R) colonies resemble S^R but are difficult to suspend in saline and produce a *deposit* in broth cultures whereas the other three types produce even turbidity They are low in virulence for mice Strains are usually iso

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Inocula do not grow on MacConkey s agar and litmus milk is unchanged Catalase oxidase phosphatase NH_3 H_2S (trace) and indole are formed in suitable media nitrates and methylene blue are reduced Acid is pro duced in peptone or tryptone media con taining glucose mannose galactose fructose and sucrose but not as a rule in rhamnose lactose raffinose melezitose starch dextrin glycogen inulin or dulcitol Glycerol may be fermented late Xylose sorbitol and manni tol are fermented by most strains except those from dogs which instead often ferment maltose and trehalose (Smith 1958) Arab inose may be fermented especially by strains from birds

Antigenic Structure

Many attempts have been made to classify *P multocida* by serologic methods Results using ordinary agglutination tests are com plicated by the common somatic antigens and the method is not now in use Four main groups designated I II III and IV have been identified using cross protection tests in mice (Roberts 1947) but the method is labori ous and can be used only with mouse patho genic strains Soluble type specific capsular antigens can be extracted by heating sus pensions to 56 C (Carter 1952) and can be adsorbed onto human type O erythrocytes which then become specifically agglutinable by homologous antisera (Carter 1955) The immunologic basis of this reaction is by no means clear since agar gel precipitin tests show that the extracts usually contain a mul tiplicity of antigens nevertheless 5 sero types A B C D and E have been thus detected Type A strains many of which are in the M (mucoid) phase when first iso lated derive chiefly from cattle fowl and pigs and are world wide in distribution they are the principal cause of fowl cholera Type B consists almost exclusively of strains from hemorrhagic septicemia in cattle and buf faloes in Asia Cultures belonging to type C occur so infrequently that generalizations as to source cannot be made Type D cul tures said to be recognizable by the char acteristic coarse agglutination of suspensions when treated with acriflavine (Carter 1957) have been found chiefly in pigs but both host range and geographic distribution are believed to be wide Type E a recently added group (Carter 1961) was obtained in cases of hemorrhagic septicemia in Africa Types A and D have been isolated from dis ease in human beings A slide agglutination technic using young (6 hour) cultures has given similar results (Namioka and Murata 1961) Satisfactory typing sera are difficult to prepare and only strains producing cap sular antigen can be typed nevertheless noncapsulated strains may be mouse patho genic and then may be typed by Roberts method Carter s types A and B correspond to Roberts II and I respectively

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Distribution and Range of Pathogenicity

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species is not usually transmitted to other species

Pathogenesis

Under conditions of stress or devitalization of the host a benignly parasitic strain may penetrate beneath the mucous membranes on which it is normally carried and may invade rapidly if transmission to other susceptible individuals takes place pathogenicity may be further increased by passage resulting in an outbreak of disease When a highly virulent strain infects an individual whose resistance is slight as in the acute form of hemorrhagic septicemia in cattle the clinical picture is that of a septicemia with high fever cardiac weakness prostration toxemia and anorexia death ensues in 12 to 36 hours The organisms are in the blood autopsy findings may be few or petechial hemorrhages may be found beneath serous and mucous membranes and in subcutaneous connective tissue the lymph nodes are swollen and sometimes hemorrhagic In less acute forms the organisms may produce a pneumonia characterized by serofibrinous exudate in the interlobular septa of the lungs and in the pleural cavity and pericardial sac or a hemorrhagic gastroenteritis Subacute and chronic infections also occur usually when a strain of low pathogenicity invades a lesion produced by some other cause for example the lesions of viral pneumonia in the lungs of swine

Immunity

Immunization of domestic mammals and birds against pasteurellosis is practiced frequently In North America vaccines are used to protect cattle from shipping fever in India cattle and buffaloes are given hemorrhagic septicemia vaccines where fowl cholera is present as in Central and Eastern European countries poultry are vaccinated These vaccines consist of bacterial suspensions killed with formalin and usually resuspended in a liquid paraffin lanoline base (Bain 1954a b 1956 1959) Their efficacy depends on correct selection of prevalent antigenic types or subtypes The rarity of human infections has made it unnecessary to develop methods for immunizing man

Hyperimmune serum prepared in horses or cattle is also available for protection of animals but its popularity has waned in recent years

Diagnosis

Painful, local inflammation or abscess formation following bites inflicted on human beings by cats and other animals deserve careful examination for *P multocida* the possibility that the organisms are present in infections should always be borne in mind especially in persons in contact with animals Five per cent blood agar is usually adequate for isolation when the material is discharged from infected wounds or sinuses, sputum pleural or cerebrospinal fluid and autopsy specimens Selective media have been devised for heavily contaminated material (Morris 1958) Inoculation of mice is also recommended as a simple method of isolation (Smith 1959)

Pure cultures of the organism are identified by appearance lack of motility at 37° C and 22° C, inability to grow on MacConkey s agar or to change litmus milk fermentation production of indole sensitivity to penicillin unusual for a gram negative rod and as a rule high pathogenicity for mice

Agglutinins may be present in the serum of infected persons (Ludlam 1944 Emson 1957) a higher titer seems to result from internal infections rather than from bite wounds If the homologous strain is not available for the preparation of suspensions several strains of different origin and if possible different serotypes should be used

Chemotherapy

Most strains are susceptible in vitro to penicillin erythromycin chloramphenicol the tetracyclines carbomycin polymyxin B cathomycin novobiocin and oleandomycin streptomycin and neomycin are less active and bacitracin and viomycin are ineffective The nitrofurans and certain sulfonamides also produce in vitro inhibition

Penicillin has proved to be adequate for the treatment of most cases of suppurative infection of the respiratory tract in humans due to *P multocida* (Olsen and Needham 1952) and is also successful in the treatment of bite wounds The prophylactic use of

penicillin in patients with such wounds especially in those cases where suturing is necessary usually prevents the development of infection (Lee and Buhr 1960) In veterinary practice penicillin and the tetracyclines particularly chlortetracycline are employed as well as the sulfonamides

Epizootiology and Epidemiology

Outbreaks of pasteurellosis in animals or birds may follow the introduction of an individual carrying a virulent strain into a susceptible herd or flock or may result when unfavorable environmental factors such as poor sanitation extreme weather or shipping lower the resistance of carrier animals *P multocida* can survive only for short periods outside the animal and the reservoir of infection is the healthy carrier

Human infections are usually acquired from animals In some cases they result from the bites of cats and dogs in others there may be a history of contact with animals but the mode of infection is obscure Most strains from the latter type of case are of serotypes A and D the most prevalent among farm livestock The organism is known to occur occasionally in the throats of healthy persons exposed to animals (Smith 1959) and may remain there for long periods later to assume pathogenic properties There is no evidence that animals suffering from pasteurellosis are a more potent source of infection for human beings than are healthy carriers

Pasteurellosis in Man

Human infections are rare but are being recorded more frequently due perhaps to increasing familiarity with the species Three principal types are recognized The first consists of bites inflicted by cats less frequently dogs and occasionally other animals Soon after the bite the area becomes painful red tender and markedly oedematous with an ascending lymphangitis and lymphadenitis (Lee and Buhr 1960) In the absence of antibiotic therapy an abscess almost always develops (Byrne *et al* 1956) Complications include necrotizing tenovaginitis and osteomyelitis (Allott *et al* 1944 Reilly and Tournier 1954 Ericson and Juhlin 1959) and in rare cases other tissues such as the meninges may become infected (Talbot and Sneath 1960) septicemia following dog bite has been reported (Williams 1960 Lee and Buhr 1960)

In the second group meningitis or brain abscess follows a head injury a nasal operation sinusitis or mastoiditis presumably in an individual carrying the organism in his nasal passages and the organism may be isolated from cerebrospinal fluid

The third category consists of infections of the respiratory tract chiefly infected bronchiectasis but also facial sinusitis pleurisy empyema bronchitis and pneumonia (Bartley and Hunter 1947 Bezjak and Mimica 1952 Olsen and Needham, 1952 Cawson and Talbot 1955 Bartley 1960) Other organs such as the heart the appendix and the kidney may also be affected In humans unlike other animals *P multocida* infection almost always results in suppuration apart from this general description is not possible symptoms and lesions varying according to the organ infected

Control Measures

Human infections probably will continue sporadically because it is improbable that infection will be eliminated from the widespread animal reservoirs Vaccination though used to a varying extent in the control of fowl cholera hemorrhagic septicemia and shipping fever of cattle and pasteurellosis of swine does not affect the carrier state and thus is unlikely to influence the incidence of human pasteurellosis Proper feeding and sanitary management are more effective and economical than treatment or biologic prophylaxis A more radical measure restocking with artificially reared pathogen free pigs now is being practiced in some farms in an attempt to eradicate certain pathogens including *P multocida*

PLAGUE
Pasteurella pestis

Plague bacilli are large gram negative elongated rods with rounded ends that stain at both poles in the parasitic stage coccus like round filamentous or other pleomorphic forms are common These nonmotile organisms grow on media containing bile

salts ferment carbohydrates only slightly (saccharose not at all) produce neither indole nor H S are milk neutral and require no accessory growth factors

P pestis causes an infection primarily of wild rodents particularly rats maintained by an insect vector the flea Man becomes a victim of the bubonic form as an aberrant interruption of the rat flea rat sequence or by handling infected wild rodents If the bubonic form becomes generalized and the lungs are secondarily affected man to-man infection leading to primary pneumonic plague ensues without intervention of the flea

HISTORY

The history of plague can be traced back almost uninterruptedly to the 3rd century before the Christian era when Dionysius told of it as a fatal disease in Libya Egypt and Syria The pandemics and the epidemics of history have been described in the works of Simpson Stricker Hirst (1953) and Pol litzer (1954) For centuries the Black Death found a highly susceptible population living in poverty congestion and ignorance in Europe and took an appalling toll justify ing all of its somber aliases and the awe in which it has always been held After the large outbreaks in Milan (1630) London (1665) and Marseilles (1721) higher sani tary standards better housing and legalized prophylactic measures contributed to its ret rogression rampant epidemic plague had disappeared from Europe Asia Minor Syria and Palestine by 1843 The last pandemic originated in Yunnan on the Burma border of China reached Canton early in 1894 from there was carried by steamer to Hong Kong and thence to Bombay within 2 years In the next 20 years India was the victim of devastating epidemics Since vessels moved in and out of Hong Kong unrestrictedly this became the focus from which plague was disseminated to seaports throughout the world It was in Hong Kong that the causal organism was discovered by a Swiss Alex ander Yersin on June 20 1894 (Ogata 1955) On July 7 1894 Kitasato announced finding an organism in plague cases but the one he described was gram positive and slightly motile

After Yersin s discovery of the bacillus India became the most fruitful center of plague work The epidemics there increased in severity and the British the German and the Russian governments sent commissions to Bombay Their reports did much to fur ther knowledge of the disease Many ob servers had noted that rats began to die before human beings were affected and that rodent and human outbreaks were somehow related Ogata in 1897 suggested tentatively that the flea might play a part in transmis sion and then a little later Paul Louis Simond working in Bombay set down the main facts about transmission epidemiology and control His hypotheses played an im portant role in the success of the antiplague experiment in San Francisco in 1903 when the U S Public Health Service put them to a test The actual part played by the rat was made clear in the reports of the British Plague Research Commission (1906 1917) physical contact with commensal rats is not followed by plague if fleas are excluded healthy rats can be kept in contact with live infected rats and with rats dead of plague without contracting the infection

That rats are not the only animals to suffer from the disease became apparent when marmotlike animals the tarabagans (*Arctomys bobac*) were found to be in fected in Transbaikalia and Mongolia in 1895 plague ridden squirrels appeared in India in 1898 the South African striped mouse (*Rabdomys pumilio*) was found to be infected in 1906 and the ground squirrel (*Citellus beecheyi*) in 1908 Today the num ber of rodents known to harbor plague is over 220 and the transition from wild to domestic species has attracted due attention in Russia South Africa the United States on the Peru Ecuador frontier and in the Argentine

Plague transmission from rats to man and among rats was studied by Ogata who in jected crushed fleas from an infected rat into mice and thereby produced plague The British Plague Research Commission work ing in Bombay (1905 1906) showed that the rat flea *Xenopsylla cheopis* transmits plague from rat to rat How the bacilli multi ply in the stomach of the flea and are then regurgitated during the sucking act of the

insect was worked out by Bacot and Martin More recently contributions to the study of fleas in plague have been made by workers in the U S Public Health Service and in the U S S R (Eskey and Haas 1940 Kartman *et al* 1958 Fenyuk 1960)

Immunization with killed organisms was initiated by Haffkine in 1896 and improved by Sokhey Vaccination with live avirulent cultures was introduced by Otten in Java and has been improved and put into practice in Madagascar (by Girard) South Africa and the U S S R Antiplague serum has been used in therapy since the time of Yersin and the potency was improved at the Haffkine Institute The therapeutic value of sulfonamides streptomycin chlortetracycline and chloramphenicol in bubonic and pneumonic plague has been established in field tests in India and Madagascar

Morphology

P pestis appear in vivo as short round coccoid or large ovoid safety pin shaped bacilli 1 5 to 2 0 microns long and 0 5 to 0 8 micron wide They are gram negative and the typical bipolar staining contrasts with the uniform color of the spherical partly autolyzed bacterial bodies In cultures bipolar staining is usually not so well seen Chainlets and larger rods are common particularly in liquid media The stain usually recommended is 1 per cent carbothionin Wayson s dye mixture (methylene blue with carbolfuchsin) or modifications of Romanovsky s or Giemsa s solutions Peculiar mold like and yeastlike serpentine forms and feebly staining irregular bladder and ring types are involution forms in environments unsuitable for growth *viz* spinal fluid of patients with chronic plague meningitis decomposed carcasses of infected animals or 3 per cent sodium chloride agar

P pestis is surrounded by an envelope particularly when cultured at 37 C It has been thought that the envelope is actually a bacterial capsule But electron microscope studies of colonies grown on collodion films over agar have shown a layer of structurally undifferentiated material of lower electron density than the bacterial cell This material lying between and among the cells in colonies is easily removed by suspending the bacilli in distilled water or by emulsifying them in saline solution before mounting them This material more abundant in virulent than in avirulent colonies is considered to be a soluble envelope or slime layer to which the nonsomatic antigens are related A structurally differentiated capsule has not been seen on *P pestis* cultured in vitro It may be incorrect to assume that the envelope observed in India ink preparations of organisms grown in vivo is in all respects identical with that synthesized in vitro (Crocker *et al* 1956)

Plague bacilli do not have flagella and are not motile

Nutrition and Cultivation

The growth of *P pestis* is slower than that of enteric bacilli Under ideal conditions the average time required for cell division is about 4 hours The lag phase can be reduced by adding the filtrate of a several day culture to the broth

Even very small inocula grow well on ordinary agar autoclaved at pH 6 with or without blood or in fluid neutral or weakly alkaline aerated media On solid agar with 0 1 per cent blood colonies appear in from 24 to 48 hours As a rule they are slimy and viscous

Smooth and rough colonies can be detected on tryptose agar to which glucose and yeast extract have been added either in obliquely transmitted light or with the aid of triphenyl tetrazolium In broth the smooth colony type produces uniform turbidity the rough brittle type accumulates as a coarse granular sediment with a completely clear supernatant

Media containing casein hydrolysate (2 5%) potassium phosphate magnesium sodium and iron sulfate with appropriate carbon sources such as xylose mannitol glucose and glycerol yield as high as 1×10^{11} viable cells per ml at 27 C By increasing the amount of magnesium sulfate and substituting cysteine hydrochloride for sodium thiosulfate and reinforcing the medium with glycine calcium pantothenate thiamine hydrochloride and biotin good yields can be obtained at 37 C (Higuchi and Carlin 1957) The minimal growth requirements are satisfied by media containing relatively

few amino acids and no added accessory growth factors (Hills and Spurr 1952 Silverman *et al*, 1954, Domaradsky, 1957) Virulent cultures grow well in the basal synthetic medium of Domaradsky and Ivanov (1957) if 0 002 to 0 004 M calcium is added they remain fully virulent for mice when injected peritoneally (Kupferberg and Higuchi 1958) Loss of virulence in aerated liquid media at 37° C is prevented by the presence of calcium or strontium and zinc salts (Higuchi *et al* 1959) or by replacing glucose by xylose, galactose or mannose (Brownlow and Wessman 1960) Avirulent strains grow without added calcium Adding magnesium oxalate to blood agar favors the growth of avirulent cells (Higuchi and Smith 1961)

Biochemical Activities

The over all fermentation pattern of glucose reveals that *P pestis* contains enzymes of both the Embden Meyerhof and the hexose monophosphate shunt pathway the latter operates during growth The CO_2 produced during fermentation arises from pyruvate (Santer and Ajl 1954 1955)

P pestis was long considered a homogeneous species but 3 biologic varieties of some epidemiologic significance have been distinguished Berlin and Borzenkov first separated 2 types The one oceanic is glycerol negative The other continental is glycerol positive and contains 2 subtypes according to whether or not it reduces nitrates to nitrites Devignat (1954) has proposed the following classification

Pasteurella pestis

var orientalis	glycerol −	nitrite +
var antiqua	glycerol +	nitrite +
var mediaevalis	glycerol +	nitrite −

The stability of *P pestis* with respect to glycerol is incontestable but the constancy of results of tests for reduction of nitrates to nitrites cannot be accepted without reservation The biochemical properties essential for classification are stable not being modified either in the laboratory or through passage from one host to another in nature (Girard 1954)

No other fundamental differences between glycerol positive and glycerol negative strains have been found and the test is primarily valuable to nosogeographers The Oriental variety (oceanic type) is found in India Burma South China Europe the United States South America Ceylon Egypt Java North Africa Senegal Sumatra Thailand and Madagascar It provoked the modern pandemic which originated in Yunnan in 1894 and spread overseas into the 5 continents The antiqua variety (continental type) seems to have been the cause in the ancient centers of pestilence in southeast Russia Central Asia Mongolia Manchuria Transbaikalia and Central Africa The medieval variety is found south of the Caspian Sea lower Volga region in the Iranian Kurdistan Turkey and Iraq

Catalase activity has been recommended (Herbert, 1949 Rockenmacher, 1949) for determining virulence of plague strains Although the mean values are different—2 23 for virulent strains 3 27 for avirulent strains —the catalase activity of each strain varies under different extrinsic conditions (Zaplatina and Borodina 1956)

P pestis produces neither H_2S nor indole and does not change milk Reduction of methylene blue and Janus green is distinct Broth cultures become alkaline and may reach pH 8 at the end of 9 weeks Hemolysis does not occur on blood plates

Resistance of the Organism

The plague bacillus in pus or sputum spread in thin layers on glass slides is killed by sunlight in 3 to 4 hours Suspensions if shaken require at least 1 hour at 65° C to be inactivated but are killed much more rapidly by acetone phenol or alcohol (Pollitzer 1954) Chloramphenicol streptomycin tetracycline or kanamycin are rapidly bactericidal in vitro In agar medium selective for *P pestis* novobiocin neomycin erythrocin and potassium tellurite allow its growth but inhibit gram negative bacteria (Morris 1958) Certain streptococci especially *Str sanguis* and *Str mitis* prevent multiplication of *P pestis* and other pasteurellae and may be responsible for its progressive disappearance from buboes as observed in human plague

P pestis remains viable in dried sputum for at least 3 months and in dry flea feces held at room temperature for 5 weeks

(Eskey and Haas 1940) Cultures and organs held in the refrigerator have retained their virulence for up to 25 years Dry ice can be used to preserve and ship specimens (Quan *et al* 1963) Suspensions of *P pestis* at pH 7 6 lyophilized rapidly with sucrose or lactose and stored at 10° C remain viable for 5 years (Heckly *et al* 1958) Virulent strains can be preserved by cultivating on 5 per cent rabbit blood agar at 37 C for 3 days sealing and storing at 4° C

Antigenic Structure

The first antigens recognized were ther molabile envelope and somatic factors iden tified by serologic absorption and precipitin tests (Schutze 1932) The envelope anti gen develops best at 37 C and withstands boiling for five minutes

The following properties characterize the most important antigens

Fraction I (FI) This material generally considered to be present in the diffusable envelope and in the capsular structure (Seal 1960) is synthesized in vitro in any medium at 37° C but not at 28 C (Keppie *et al* 1958 1960) Quantitative pre cipitation tests are favored for antigen estimation the hemagglutination test is also useful and the complement fixa tion test less so The antiserum must be absolutely specific purified FI is used as antigen The agar diffusion technic is find ing increasing application in antigen analysis (Chen *et al* 1952)

Toxin Fresh suspensions of live virulent plague bacilli (2×10^8 in 0 5 ml) injected intravenously into mice are fatal within 4 to 12 hours Supernatants of autolyzed cul tures or saline extracts of acetone-dried or ganisms yield by fractional purification a product with a mouse LD_{50} of 0 1 to 0 3 mcg (Ajl *et al* 1958 Spivack and Karler 1958) This preparation is relatively free from Fraction I but immunodiffusion tests show the presence of 1 to 3 contaminating antigens Abou 0 05 per cent of the weight of plague organisms is toxin The purified material has a molecular weight of 74 000 and yields on hydrolysis 18 amino acids and calcium sodium and magnesium but no unusual components (Bent *et al* 1957) Incubation for 4 hours at 56 C partially destroys it the destruction is complete at 75 C for 5 minutes It is stable over ranges of pH from 5 to 8 Formalin (0 1%) con verts it into a toxoid that retains its antigen icity

Purified toxin sensitizes tannic acid treated erythrocytes and this provides a useful pro cedure for determining serum antitoxin levels This can be done also by complement fixation

The toxin is highly lethal for mice and rats but not for guinea pigs rabbits or pri mates Hyperimmunization of rabbits and monkeys with living virulent or avirulent plague bacilli gives rise to antibodies 10 LD_{50} of toxin can be neutralized by 0 01 to 0 0025 ml of serum when tested by the in traperitoneal route The neutralizing anti bodies react quantitatively with the pure toxin in flocculation systems but the anti body toxin complex is not stable On intra venous administration, dissociation occurs Antitoxic serum devoid of envelope anti bodies does not influence infection with toxic virulent strains in mice nor does it protect the vascular system against the toxin released in the course of infection Prolonged immunization of mice with partially purified toxin may confer some protection against *P pestis* infection however complement fixing and hemagglutinating antibodies against en velope antigen are commonly found in the animal after such treatment.

The toxin acts mainly on the peripheral vascular system, causing hemoconcentration shock and parenchymal injury in the liver and the kidneys On intradermal administra tion local edema is often followed by tissue necrosis Observation that the toxin mole cule can inhibit mitochondrial respiration prompted Ajl and Rust (1960) to test its effects on the heart Electrocardiograms re vealed changes before detectable alterations in blood pressure or hematocrits However rats succumbing to infection after injection of a low dose (10^3 organisms per rat) failed to show cardiac changes at any stage from infection to death (Rust *et al* 1960)

Other toxic substances or complexes have been isolated from *P pestis* A lipopolysac charide lethal for mice and guinea pigs in relatively large doses was extracted in hot 45 per cent phenol from acetone-dried viru lent or avirulent organisms (Davies 1956) The LD_{50} of this material for mice guinea

pigs and rabbits was 10 2 and 5 mg respectively Seven mcg per Kg was pyrogenic in rabbits raising body temperature to 40 6° C in 2 hours Active or passive immunization with this substance did not protect mice or guinea pigs against plague infection

Another toxic protein isolated from cells grown in vivo or for 2 to 3 hours in vitro at pH 6 and 37° C is highly dermatotoxic for rabbits and cytotoxic for monocytes (Bichowsky-Slomnicki and Ben Efraim 1963) It is unstable and disappears from killed cells after storage for a few days Like the lipopolysaccharide antigen it is produced by both avirulent and virulent strains All efforts to demonstrate a toxin produced in vivo similar to that in anthrax have failed

Other Antigens V and W antigens are found when virulent plague bacilli are grown in tryptose digest of meat broth in tubes rotated slowly at 37° C, but not at 28° C for 3 hours When organisms so prepared are tested in gel diffusion plates against homologous antiserum absorbed with avirulent strain TS similarly prepared a single line of precipitation is formed Since this line is given by all virulent strains and is the only antigenic difference detectable between these and avirulent strains it has been designated Vi antigen If organisms are incubated under the same conditions for 5 or more hours a second antigen is produced by all strains capable of producing Vi antigen The two antigens are designated V and W (Burrows and Bacon 1954 1956 Burrows 1963) The conditions for producing these antigens are very critical Gluconate may improve the yield (Lawton *et al* 1960) V antigen is not detectable in the unconcentrated supernatant but is in the sedimented organisms V antigen is a protein with a molecular weight of 90 000 W is a lipoprotein with a molecular weight of 145 000 Both are destroyed in 10 minutes at 80° C and inactivated by treatment with trypsin but resist the action of periodate The use of rabbit antiserum containing only V antibody or only W antibody has shown that V antibody but not W can protect mice against plague (Lawton *et al* 1963) High resolution diffusion plates reveal that the apparent single V and W lines in reality consist of double lines suggesting that each may consist of 2 components (Burrows 1963)

Investigation of the change in electrophoretic mobility under particular conditions of pH and temperature of *P pestis* culture has revealed a new antigenic component called pH 6 antigen (Ben Efraim *et al* 1961) This pH 6 antigen is synthesized in vivo in rabbits and mice, and the rate of mortality is increased in groups of mice inoculated with virulent *P pestis* containing the pH 6 antigen (Ben Efraim *et al*, 1961), suggesting that the pH 6 antigen may be involved in the pathogenesis of plague The well known damage to tissues induced by whole *P pestis* may well be attributable to pH 6 antigen

Polysaccharides Polysaccharides are found in 4 to 7 day-old cultures of *P pestis* Girard and Sandor failed to obtain from plague bacilli a glucolipoid complex similar to that of other gram negative microorganisms However extraction of dried *P pestis* with saline to remove the soluble protein followed by aqueous phenol extraction and ethanol precipitation yields a lipopolysaccharide (1 6% N and 2 2% P) (Chen 1952 Chen and Meyer 1954)

Pesticins *P pestis* produces a bacteriocin like substance of protein nature called pesticin (Ben Gurion and Hertman 1958) Pesticin production and bacterial growth inhibition is highest near 37° C Pesticin sensitivity is restricted to strains of *P pseudotuberculosis* belonging to the serologic Group A (Burrows and Bacon 1960) In a survey of many strains of *P pestis* 3 of them (TRU A_{12} Java) failed to produce pesticin but did form another pesticin called pesticin II (Brubaker and Surgalla 1962) Many other strains produced neither pesticin I nor II (Smith and Burrows 1962) The complex pattern of pesticin production and sensitivity so far described is further complicated by a metabolic product inhibiting pesticin I Pesticins may be involved in certain growth suppression selective for either virulent or avirulent cells

Distribution Virulence and Pathogenicity

In bubonic plague in man *P pestis* can be cultured or seen in smears of fluid from primary vesicles and in the gelatinous edema fluid surrounding or within lymph nodes Bacteremia—the rule even in mild bubonic infection—may disappear as early as the 2nd

day or it may persist for 10 days in severe infections At autopsy *P pestis* is regularly found in heart blood lymph nodes or spleen but especially in bone marrow and secondary pneumonic lesions In primary pneumonic plague the bacilli are in sputum before they can be detected in the blood

Over 200 species of wild rodents are now known to be naturally infected or are strongly incriminated hosts for *P pestis* (Pollitzer and Meyer 1961) The cosmopolitan rats (*Rattus norvegicus R rattus* subspp) and commensal mice (*Mus musculus*) are equally important hosts Other mammals are suspected to be naturally susceptible Cats and dogs are moderately susceptible (Pollitzer 1954) chickens are not absolutely resistant to massive experimental infection (Hoessly 1954) It has been claimed that sheep and camels can be infected (Fedorov 1960)

The rodents usually used in experimental work—guinea pigs white mice multimammate mice cotton rats rabbits rats—are all susceptible to plague Experimental plague can be produced in guinea pigs by any route A few virulent organisms can establish infection in most of these animals but there may be seasonal and genetic variations Guinea pigs are desirable subjects for diagnostic work because the lesions that develop are so characteristic White rats are not as susceptible as other *Muridae* and lesions produced in them are not as characteristic as those in the guinea pig

Virulence This term is used to denote the ability of different strains or isolates to produce death from plague measured in LD_{50} The virulence of most of the tested strains freshly isolated from human or rodent infection has been high Barber found that 6 of 9 guinea pigs and 2 of 12 monkeys receiving 1 freshly isolated virulent plague bacillus died of plague Some strains that have lost their virulence as the result of cultivation on inadequate media will regain it on passage through susceptible animals Avirulent strains with qualities that recommended them for use in vaccines have been isolated from cultures held in deep agar slants for 5 to 6 years at 0° ± 4° C

In chick embryos highly virulent plague strains proliferate freely avirulent strains sparingly The former in small numbers kill the embryo the latter in sublethal doses persist in embryo organs until hatching and sometimes for 3 to 4 days after (Buddingh and Womack 1941 Jawetz and Meyer 1944b)

Virulence is associated with specific antigens According to Burrows and Bacon (1955 1956) and Burrows (1963) fully virulent strains must have the ability (1) to elaborate envelope antigen (2) to develop resistance to phagocytosis (mouse polymorphonuclear cells) in the absence of visible capsulation (3) to synthesize antigens V and W (4) to produce pigmented colonies on a defined medium containing hemin (5) to synthesize purines and (6) to be highly toxic

Fraction I a surface antigen protects in vivo grown organisms against phagocytosis The envelope seems to be required for full virulence but certain noncapsulated strains that still produce V and W antigens retain considerable virulence (Burrows and Bacon 1958) The F± (so-called noncapsulated) *P pestis* of reduced pathogenicity does not produce fatal infection in the guinea pig by the intraperitoneal route but infects this rodent by the intradermal route As few as 10 cells induce skin lesions bubo and fever but all animals survive even following infection with 4 500 cells By contrast a single bacterium of the fully virulent encapsulated FI+ strain causes lethal infection in the guinea pig after intradermal injection The capsule seems much more essential for lethality than for infectivity (Donovan *et al* 1961)

The resistance to phagocytosis associated with the FI and VW antigens in virulent *P pestis* at 37 C does not prevent their ingestion by fixed macrophages of the reticulo-endothelial system (Fukui *et al* 1962) Evidence presented by Janssen (1963) strongly suggests that the ability to survive and multiply within the phagocytic cell is the major determinant of its virulence not the ability to resist phagocytosis

Antigens other than FI and other factors are involved in virulence The consistency of the findings that all virulent strains tested are VW+ and that no VW— strains have proved to be virulent is presumptive evidence that the character VW+ plays a part in the ability of *P pestis* to survive and multiply in

phagocytes Since the antigens designated 4 and pH 6 contribute to the survival of *P pestis* in vivo, they may also be factors in virulence

Virulent strains grown on a particular de fined medium containing hemin form dark brown colonies (Burrows 1955) (P+) others produce whitish or straw-colored col onies in the same medium (P—) The pig mentation results from the absorption of hemin from the medium Loss of the deter minant P+ decreases virulence for the guinea pig more than for the mouse Simultaneous injection of iron salts restores full virulence for mice but not for guinea pigs (Jackson and Burrows 1956) Not only does injected iron reduce the LD_{50} to the level of that of virulent strains but it also permits the pro duction in vivo of the large bacillary popu lations characteristic of virulent infections Whether the character P+ is directly con cerned in virulence or more likely is asso ciated with some other property it is in practice a useful and easily scored character for following population changes resulting from loss of virulence and for characteriza tion of strains in the laboratory For exam ple if a strain scores as P— it will be of low virulence regardless of any other deter minant The well known avirulent strain E V 76 used safely in large scale immuni zation of man in Madagascar is FI+ and VW+ but P—

Various nutritional factors differentiate virulent from avirulent *P pestis* Purine dependent mutants of virulent strains fail to produce disease because of the lack of free purine in the host but do so when purine is injected into the mice simultane ously with the bacilli (Burrows 1955)

All fully virulent strains are toxic (T+) but most avirulent strains also produce a protein that is very toxic for mice and rats less so for monkeys and rabbits and almost nontoxic for guinea pigs Laboratory strains thus far recognized as T— are deficient in one or more other virulence factors

Pathogenesis Essential for the develop ment of bubonic plague in man is the primary rat flea rat transmission cycle The flea be comes infected by taking blood from a plague infected rat in the terminal bac teremia rat blood may contain 10^7 bacilli per cu mm (Douglas and Wheeler 1943) These bacilli multiply in the midgut of the flea and massive infection develops in the proventriculus, blocking the pharynx and the esophagus When the flea attempts to take its next blood meal from 25 000 to 100 000 bacilli are regurgitated via the insect s pro boscis into the skin or the capillaries of the new mammalian host Other rodents may re place the rat in this cycle

The infection of man is an offshoot rarely are there enough bacilli in human blood to infect fleas Fleas ordinarily infesting man are usually not good plague transmitters but under certain circumstances human fleas can transmit bubonic plague from man to man directly or indirectly when the infesta tion is extremely heavy (Swellengrebel 1953)

When plague bacilli become localized in the lungs and produce pneumonia droplets from the respiratory tract are highly infec tious Then primary pneumonic plague may spread readily from man to man and a true epidemic occurs There are several other less probable means of infection—inhalation of dust or of infected flea feces and eating of undercooked plague infected marmots (in Manchuria)

P pestis may enter the body via the blood the skin the conjunctiva or mucous mem branes of the respiratory or the digestive tracts

The plague bacillus injected into the skin may be held up at the site the local vesicle or pustule represents the first line of defense This symptomless form reflects considerable immunity Observations among people in plague areas of Madagascar (Payne *et al* 1956) in Ovamboland and Peru (Meyer 1964) and surveys of rodents in endemic plague areas in the United States (Hudson 1963 Randall 1963) suggest that this form may be more common than it had been thought to be

If *P pestis* passes the skin barrier it next reaches lymph nodes which enlarge and are embedded in a gelatinous periglandular in flammatory edema The infection may be arrested at this stage with only mild con stitutional symptoms (pestis minor) If the bacilli pass this second line they reach the secondary lymph nodes draining the area

of inflammation and small numbers pass into the bloodstream and from there to the spleen the liver and other lymph nodes An inter play between antibodies and fixed tissue leukocytes and possibly other factors may limit this bacteremia to showers of organ isms Nevertheless generalized infection in many parts of the body creates grave con stitutional symptoms If the immunity is in adequate or has been damaged by toxins plague bacilli not only multiply intravascu larly but are constantly washed into the circulation from the spleen and bone mar row causing septicemic plague In the course of bacteremia bacilli may localize second arily in the skin the lungs and various other organs Wherever plague bacilli multiply to enormous numbers coagulation necrosis oc curs These abscesses undergo slow resolu tion and may contain viable bacilli for many weeks

The LD $_0$ is much greater when tested by the respiratory route than by the sub cutaneous Two types of plague can de- velop in the respiratory tract of mice or guinea pigs depending on the size of the particle introduced Small particles initiate bronchopneumonia which leads to septi cemia and death with pulmonary edema Large particles establish septicemia, and death results more quickly without pneu monia (Druett *et al* 1956) The portal of entry may be the lymphatic tissues of the oropharynx in man this type of infection has been described as tonsillar plague (Meyer and Larson 1960) Primary fatal pneu monic infection in monkeys can be brought about by intratracheal instillation of 100 virulent *P pestis* inhalation of about 20 000 cells induces a chronic pneumonic process in which virulent bacilli are recoverable for 40 days (Ransom and Krueger 1954) The pathogenesis of secondary lung involvement after bubonic infection is not clearly under stood It may be that emboli from focal lesions in lymph nodes liver or spleen lodge in the pulmonary capillaries (Meyer 1957)

IMMUNITY

The immunity that follows infection is rel ative Patrick Russel recorded 28 reinfec tions in 4 400 cases

Resistance of wild rats or squirrels to ex perimental plague depends on the extent of endemic disease in the localities they have inhabited (Meyer, 1942) In India where plague had been prevalent only 7 9 per cent of several hundred rats tested were suscep tible to experimental plague (Sokhey and Chitre 1937) But in Madras City where the rodent population had not been exposed 91 7 per cent were susceptible

Serum plasma and other body fluids of animals that have recovered from plague or been immunized contain a variety of anti bodies but they can neither lyse nor destroy *P pestis* in vitro or in vivo in the absence of phagocytic cells Whole blood of immune animals destroys many more plague bacilli than does that of normal animals Immune animals fix the bulk of bacilli at the site of injection however the protection does not depend on lymphatic blockage and deposi tion of fibrin network In the animal with an acquired immunity the bacilli are readily phagocytized and lysed within polymorpho nuclear leukocytes Excessive soluble anti gen may block the action of the antibodies and endotoxins liberated by the lysed or ganisms may paralyze the immunity (Meyer 1950 Cavanaugh and Randall 1959)

In mice only Fraction I and VW anti bodies acting together have protected ef fectively against virulent organisms Guinea pigs have been protected against virulent challenge with formalized whole organisms with low doses of Fraction I (1 to 50 mcg) in adjuvant and with living *P pseudotuber culosis* (Spivack *et al* 1958 Lawton *et al* 1960) Alum and oil adjuvants greatly in crease the potency of bacillary suspensions or extracted purified Fraction I and later booster inoculations

Prevention of plague by vaccination has been studied ever since Haffkine in 1896 made extensive animal and human experi ments with heat killed broth culture anti gens The attack rate was not impressively reduced after administration of one dose of Haffkine antigen But in sulfa treated pa tients the mortality rate of those immunized with Haffkine broth was less than half that of the unimmunized group (Patel and Rebello 1948)

Experiments in guinea pigs and certain commensal rats led French and Dutch work

ers to conclude that vaccination with killed organisms would not protect man In contrast field tests in Java and Madagascar with vaccines composed of living avirulent bacilli have shown promise In endemic areas where the native populations are heavily exposed at times, a preparation that can be given in a single dose has administrative and economic advantages The annual reports of the Pasteur Institute at Tananarive, Madagascar, have shown a striking decline in the number of cases—from 3 035 in 1935 to 14 in 1954 1955 under the impact of 600 000 to 800,000 annual vaccinations with living avirulent plague vaccine

Living vaccines which contain all permissible antigenic components and which produce hyperplasia in the reticuloendothelial system confer the highest protection against natural infection (Korobkova and Samoilova 1962) Unfortunately they may cause unpleasant reactions at the usual dose (1×10^9 organisms) The systemic reactions are severe following intramuscular injection Subcutaneous injection causes local infiltrations and pustules which may last up to 7 days (Koslov *et al* 1960) A single inoculation of the living attenuated strain EV76 elicits demonstrable envelope antibody and antitoxin in relatively few persons, but annual inoculation progressively enhances the immune response (Girard 1955) A single subcutaneous inoculation with live or killed vaccine does not protect against aerogenic infection Some persons who have been vaccinated repeatedly have died of plague

In the United States attention has been focused on the development of immunogens consisting of killed bacilli At first Fraction I was tried but in the few trials on volunteers and experiments on animals a suspension of virulent plague bacilli killed with formalin to convert toxin to toxoid produced milder local and systemic reactions Adjuvants enhance the immunogenicity of killed vaccines but since oil adjuvants are regarded with disfavor by the medical profession alum-coated preparations are being studied An adequate dose of formalin killed alum-coated suspensions of virulent *P pestis* (1 and 0 5 *ml* or 2 5 and 1 25 $\times 10^9$ *P pestis*) in 2 doses 1 month apart increases resistance without causing more than occasional local or systemic reactions Booster doses are required to raise the mouse protective antibodies to levels similar to those in the serum of patients who have recovered from plague Reinoculation every 3 to 6 months is essential to maintain immunity in man Low hemagglutinin titers persist up to 10 years after the initial immunization A booster inoculation with a very small dose of killed vaccine (0 2 ml or 5×10^9) is sufficient to bring complement fixing antibodies and hemagglutinins to the level found in plague convalescents

Whether the envelope antigen in adjuvant or killed vaccines is as protective as live attenuated vaccines cannot be answered directly Natural exposure is becoming increasingly rare because the disease is receding Measurement of the antibody response is therefore the only known way to evaluate immunization

Yersin and his colleagues were the first to demonstrate that serum from rabbits immunized with inactivated cultures protects other animals against infection These results established the principle of passive immunization and gave hope for serum prophylaxis and serum therapy The effectiveness of serum has been proved repeatedly the mortality rate among the treated in one series was about 28 per cent in contrast with a rate of 58 per cent in the untreated patients

DIAGNOSIS

Diagnosis is likely to be missed in sporadic cases Early diagnosis is of vital importance to the patient and the attending staff as well as to his family and community A typical case of severe bubonic or septicemic plague presents a characteristic picture sudden onset high temperature *rapid* pulse white coating of the tongue nervous symptoms varying from restlessness to great prostration and fatigue bloated appearance and conjunctival suffusion slurred speech and staggering gait apathy and mental confusion Eventually there is pain in the groin the armpit or the neck where the bubo appears *Intense pain directs the patient* s attention to the inflamed node which may remain small hard and tense but more frequently enlarges to the size of a walnut or a goose

egg and is embedded in boggy edema In the septic variety nervous and cerebral symptoms supervene with striking rapidity although the temperature is rarely above 100 F epistaxis hematuria and involuntary evacuation appear in rapid succession Pneumonic plague begins with rigor malaise severe headache nausea vomiting and general pain temperature of 102° to 105 F difficult and hurried breathing cough and expectoration The sputum watery and frothy becomes blood tinged but is rarely viscid or rusty as it would be in acute pneumonia Diagnosis requires laboratory assistance The specimens should be collected before streptomycin or tetracycline treatment is begun

Laboratory Diagnosis This subject has been well covered by an international group of plague workers (Baltazard *et al* 1956 American Public Health Association 1964)

The bubo should be punctured in its early stages with an 18 gauge needle mounted on a well fitted 5 to 10 ml syringe and a little gelatinous edema fluid aspirated The person taking the specimen should wear rubber gloves and mask The skin over the bubo is painted with iodine and the puncture wound is disinfected with alcohol Care must be taken in expelling the few drops of fluid for culture or staining not to spray bacilli into the atmosphere Diagnosis can be made from the characteristic polychromatic stain and from the small delicate colonies which develop at 30 C in 24 to 48 hours However contaminants such as *E coli* or salmonella can also give a bipolar stain The culture can be identified quickly by specific bacteriophage agglutination tests and preferably by fluorescent antiplague globulin on smears fixed by heat (Winter and Moody 1959)

For agglutination tests the bacterial growth is emulsified and maintained in a 0 45 per cent saline solution containing 1 per cent formalin for 30 minutes to 2 hours at 37 C When the suspension free from precipitates is added to the serum flocculent agglutination usually appears within 2 hours at 37 C

Final identification is made by biochemical tests and guinea pig inoculations Infection can be accomplished by rubbing some of the culture intracutaneously or by injecting subcutaneously into mice or guinea pigs a few drops of a heavy suspension The animals usually die within 3 to 8 days with characteristic local and general lesions

Blood cultures must be made from at least 5 ml of citrated blood Part of the blood is placed in enriched cystine broth and the rest is distributed on several blood agar slants or plates More than 10 colonies indicate severe bacteremia

Sputum should be examined both microscopically and culturally on blood gentian violet (1 70 000) plates or the selective medium developed by Morris (1958) There is usually no difficulty in recognizing the plague bacilli which are present in great numbers

Every state in the U S authorizes physicians or health officers to demand an autopsy if plague is suspected Plague infected material must be shipped in properly prepared containers with double screwtops The material should include heart blood portions of bubo spleen and bone marrow The precipitin test or a modification with fluorescent antibody can be used to examine decayed or mummified carcasses during field investigations of suspected plague outbreaks among wild rodents (Hudson *et al* 1962) The local or state health officer must be notified the moment the diagnosis is suspected

Treatment

Treatment has progressed from almost useless to highly successful within the last decade Chemotherapy has displaced serum and bacteriophage therapy Reduction in mortality rate moderate cost and oral route of administration highly recommend sulfonamides and extensive field trials in India give preference to sulfadiazine Their value in preventing contagious primary pneumonic plague is not known Combined use of sulfonamides and specific plague antiserum gives results superior to those produced by either agent alone

Penicillin is useless Streptomycin is the best therapeutic agent thus far tested It may cure mild infections within 48 hours but should be continued if the clinical course warrants Chlortetracycline and chloramphenicol therapy is complicated by relapse if not continued for 7 to 10 days In India

ers to conclude that vaccination with killed organisms would not protect man In contrast field tests in Java and Madagascar with vaccines composed of living avirulent bacilli have shown promise In endemic areas where the native populations are heavily exposed at times a preparation that can be given in a single dose has administrative and economic advantages The annual reports of the Pasteur Institute at Tananarive Madagascar have shown a striking decline in the number of cases—from 3,035 in 1935 to 14 in 1954 1955 under the impact of 600 000 to 800 000 annual vaccinations with living avirulent plague vaccine

Living vaccines which contain all permissible antigenic components and which produce hyperplasia in the reticuloendothelial system confer the highest protection against natural infection (Korobkova and Samoilova 1962) Unfortunately they may cause unpleasant reactions at the usual dose (1×10^9 organisms) The systemic reactions are severe following intramuscular injection Subcutaneous injection causes local infiltrations and pustules which may last up to 7 days (Koslov *et al* 1960) A single inoculation of the living attenuated strain EV76 elicits demonstrable envelope antibody and antitoxin in relatively few persons, but annual inoculation progressively enhances the immune response (Girard 1955) A single subcutaneous inoculation with live or killed vaccine does not protect against aerogenic infection Some persons who have been vaccinated repeatedly have died of plague

In the United States attention has been focused on the development of immunogens consisting of killed bacilli At first Fraction I was tried but in the few trials on volunteers and experiments on animals a suspension of virulent plague bacilli killed with formalin to convert toxin to toxoid produced milder local and systemic reactions Adjuvants enhance the immunogenicity of killed vaccines but since oil adjuvants are regarded with disfavor by the medical profession alum-coated preparations are being studied An adequate dose of formalin killed alum coated suspensions of virulent *P pestis* (1 and 0 5 ml or 2 5 and 1 25 $\times 10^9$ *P pestis*) in 2 doses 1 month apart increases resistance without causing more than occasional local or systemic reactions Booster doses are required to raise the mouse-protective antibodies to levels similar to those in the serum of patients who have recovered from plague Reinoculation every 3 to 6 months is essential to maintain immunity in man Low hemagglutinin titers persist up to 10 years after the initial immunization A booster inoculation with a very small dose of killed vaccine (0 2 ml or 5×10^8) is sufficient to bring complement fixing antibodies and hemagglutinins to the level found in plague convalescents

Whether the envelope antigen in adjuvant or killed vaccines is as protective as live attenuated vaccines cannot be answered directly Natural exposure is becoming increasingly rare because the disease is receding Measurement of the antibody response is therefore the only known way to evaluate immunization

Yersin and his colleagues were the first to demonstrate that serum from rabbits immunized with inactivated cultures protects other animals against infection These results established the principle of passive immunization and gave hope for serum prophylaxis and serum therapy The effectiveness of serum has been proved repeatedly the mortality rate among the treated in one series was about 28 per cent in contrast with a rate of 58 per cent in the untreated patients

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builds his homes so that rats cannot nest or obtain food (3) In certain environments more frequently in cold countries inter human parasitism as pneumonic plague spreads by droplet infection and causes explosive epidemics as it did in Manchuria in 1910 to 1911 and 1920 to 1921 (60 000 and 8 502 victims respectively) (Pollitzer 1954 Hirst 1953) This form of parasitism may also appear as bubonic plague for example in the rural regions of Morocco and the Valley of the Kurdistan Mountains where the human flea acts as the vector

The seasonal spread is influenced by temperature and humidity The disease tends to occur during summer months in cooler climates and in the spring months in the hot dry climates of the subtropics in tropical countries where the temperature is fairly constant throughout the year the incidence follows the humidity curve

As the rodent population in urban areas is reduced by a rampant epizootic and the fleas seek new hosts persons of all ages and both sexes may contract bubonic plague There does not seem to be any racial predilection Doctors and nurses who care for patients with pneumonic plague and other contacts including funeral attendants are liable to contract the pneumonic form Strains of *P pestis* recovered from man rats wild rodents and fleas from every corner of the earth are biologically identical and remarkably homo geneous in virulence and infectiousness Vari ations in the severity of pneumonic epi demics are not attributable to a specific pneumotropism

Endemic plague foci maintained by bur rowing hibernating rodents such as tara bagans *Citellus* varieties spermophils and mice are found in the bush the deserts and the mountains of Manchuria the Buriat Mongol Republic Transcaucasia and South eastern Russia foci exists in South Africa among gerbils and multimammate mice (Davis 1953) in Argentina among *Micro cavia graomys* and in squirrels along the Peruvian Ecuadorian frontier In the United States rural plague was discovered in 1908 and since 1934 epizootics have broken out among squirrels prairie dogs rabbits and pack rats in 15 western states Enzootic and epizootic plague exists among at least 50 rodent species Interest has shifted from the large colonial rodents such as ground squir rels to the small inconspicuous native field voles and mice such as *Microtus* and *Pero myscus* Co-existence of these small rodents with domestic rats in the security of human habitations and the prevalence of unrecog nized enzootic plague in the wild rodent populations pose a continuous potential threat (Kartman *et al* 1962) Intimate as- sociation of cottontail rabbits with wood rats white footed mice and other permanent hosts of plague led to the exchange of the parasite and then to man (Kartman 1960) Epi zootics among the large gray rats (*Rattus norvegicus*) the black domestic rats (*Rat tus rattus alexandrinus*) and occasionally others such as house rats (*Rattus grisei venter*) and field rats (*Rattus diardii* in Java and *Rattus hawaiensis* in Hawaii) are in fluenced by natural and acquired suscepti bility the density of the rat population and the circumstances which bring the species into close association with man

Plague may be introduced into a rat pop ulation by spread from one section of a city to another by spread at a distance through transportation of rats and rat fleas along lines of communication with merchandise or by partial migration of rodents as in India (Sharif 1951) Unless there is a consider able population of infected *Rattus rattus* plague is never transmitted to man to any serious extent In San Francisco in 1907 27 (2 69%) of 1 002 live rats examined were infected 56 human cases of plague with 25 deaths were reported during that time By January after a rigorous control campaign despite an incidence of 1 11 per cent among another tested group of rats of the city only 2 cases of human plague were listed Rats surviving the epizootics have ac quired resistance to infection High immunity rates for example over 50 per cent in San Francisco and over 90 per cent in Bombay limit the spread and may even lead to the eventual disappearance of plague Epizootics cannot arise until a new generation of sus ceptible rats has grown up

Plague in order to maintain itself in focal rural areas requires resistant wild rodents to survive epizootics They probably per

streptomycin dramatically reduced mortality rates from 50 to 10 per cent in both bubonic and septicemic plague The most spectacular effect is seen in pneumonic plague formerly irremediable (McCrumb *et al* 1953) About 10 hours after the beginning of treatment virulent *P pestis* disappear from the sputum and the risk of contamination of the attendant staff and those associated with the sick person is thus reduced Very rarely strains develop resistance to streptomycin within 48 hours after treatment of bubonic and pneumonic plague Chloramphenicol is an excellent alternate In the treatment of the bacteremic stage with massive doses of streptomycin the rapid destruction of *P pestis* may release large quantities of toxin thus causing sudden death The recommended treatment schedule for streptomycin is an initial injection of 2 Gm followed by 1 Gm per day for 10 days or longer (Meyer 1957)

EPIDEMIOLOGY AND ECOLOGY

Plague is endemic in certain parts of India Kurdistan Burma Java China Madagascar and South Central and East Africa It occurs sporadically in Egypt North Africa (Tunis) Iraq Iran Siam and Indo-China (Pollitzer 1954) The following number of cases have been reported by WHO in 1962 Europe 1 Americas—Ecuador 326 Brazil 36 Venezuela 1 Africa—Central Africa 5 South West Africa 80 Cape Provinces 4 Madagascar 28 Asia—India 697 Burma 49 Viet Nam 29 Local rat epizootics occasionally accompanied by a few human cases have been noted in European seaports from time to time the most recent ones being on Malta and Sardinia

The plague foci in America constitute a potential source of epidemic plague The United States has known rat and human plague since the beginning of the century San Francisco (1900 and 1907) the Gulf States—Louisiana Texas and Florida (1914 to 1920)—and Los Angeles (1924 to 1932) were affected in turn After a 20 year hiatus the rat disease erupted in Tacoma Washington in 1943 and 1944 Rural endemic murine foci seem to have disappeared from Maui and Hawaii Pockets of enzootic and epizootic plague have been recognized in ground squirrels chipmunks, prairie dogs wood rats and harvest mice as far east as Kansas Oklahoma and Texas Since 1908 70 cases (45 deaths) have been traced to wild rodents in these areas (Link, 1955 Kartman *et al* 1958)

Differences in the race and the sex incidence of bubonic plague cases as well as occasionally observed increased incidence of the disease among certain occupational groups (e g dock workers grain handlers) are due merely to differences in exposure Plague has been said to be rare in young children but of 2 994 bubonic and septicemic cases in Madagascar 104 (3 1%) were in children up to 2 years of age

The modern trends of epidemiology deal with the ecology of plague rather than with the epidemiology of the disease (Pollitzer and Meyer 1961) Two fundamentally different forms of the ecology of plague must be considered (1) bubonic or zootic plague produced usually by the bite of plague infected insect vectors mainly rodent fleas and (2) primary pneumonic or demic plague due to spread from man to man The basic prerequisite for the single cases outbreaks or epidemics is maintenance in the rodent population Periodic epizootics exterminating a variable proportion of the rodents offer opportunity for man to become infected either directly or, more often through fleas

The usual life of the plague bacillus is that of a parasite of wild rodents in the areas never touched by man Swellengrebel recognizes 3 classes (1) Wild parasitism sylvatic plague plague of wild rodents or rural plague In this milieu human infections occur rarely and only among persons such as hunters exposed to rodent bites and occasionally to fleas Through exchange of ectoparasites the plague bacillus can extend its range to commensal rodents particularly rats and mice (2) Domestic parasitism or urban plague is eminently suited for passing the plague bacillus to man through fleas giving rise to bubonic plague It occurs in densely populated unsanitary areas spreads along overland routes and crosses oceans in ship cargos Primarily bubonic it tenaciously fixes itself to human habitations and spreads as outbreaks but it disappears when man

and in some the disease has ceased to be manifest for the present History teaches that plague has often shown a spontaneous de cline but has flared up again The present low incidence might really reflect natural periodicity of the infection The possibility of future pandemics cannot be excluded therefore the disease should be given con tinued attention by those interested in global public health

PASTEURELLA PSEUDOTUBERCULOSIS*

The large gram negative elongated *P pseudotuberculosis* organisms are pleo morphic sometimes occurring in chains Biopolar staining is inconstant Motility when they are cultivated at 18 to 22 C is due to 1 or 2 parapolar or rarely 3 to 6 peritrichous flagella The organisms grow in media containing bile salts and in amino acid solutions in the absence of accessory growth factors they produce no gas in carbohy drates and no indole and litmus milk even tually becomes alkaline The antigenic rela tionship to salmonella suggests classification with the *Enterobacteriaceae* rather than the *Parvobacteriaceae*

Human infections may take a typhoidal or an enteric form The recent addition of nearly 400 infections of the latter type to the previous list of only 16 reported cases places pseudotuberculosis in a more im portant position as an infection of man *P pseudotuberculosis* can also infect rodents and birds

Synonyms are *Streptobacillus pseudotuber culosis rodentium* (Preisz) *Bacterium pseu dotuberculosis rodentium* (Lehman and Neumann) *Bacillus parapestis* (Lerche) *Bacterium peseudotuberculosis rodentium* Preisz (Schutze) *Malleomyces pseudotuber culosis rodentium* (Pribaum)

History

The organism was first isolated by Malas sez and Vignal in 1883 on inoculation of guinea pigs with material from a subcutane ous tubercular lesion on the forearm of a child who had died of meningitis The animals developed nodules which though histologically similar to those in tuberculosis contained masses of coccobacilli The agent is encountered in epizootics among barnyard fowl cage birds cats and monkeys

Morphology

P pseudotuberculosis varies in shape and size according to conditions of growth it may be coccal or ovoid less than 1 μ long or it may form rods 0 5 micron by 1 5 to 5 0 microns with rounded ends either singly in short chains or filaments It is gram negative and seldom takes bipolar stain in contrast with *P pestis* At room temperature even in repeated transfers all typical strains are motile and swarm Examination by light and electron microscopy reveals 1 or 2 mostly parapolarly located flagella 4 or 5 times longer than the bacterium Single rods occasionally show peritrichous flagella Smooth and motile colonies develop best at temperatures between 20 and 30 C (Knapp 1956) Neither spores nor definite capsules are formed though at 22 C a viscous layer (envelope) may be seen in India ink preparations

Cultivation and Biochemical Activities

P pseudotuberculosis grows aerobically in ordinary media Cultivation at 37 C ac celerates dissociation Growth occurs from pH 6 to 8 but acidity favors R dissociation The smooth to slimy light transparent colonies reach a diameter of 2 to 3 mm on the 2nd day on agar medium with or without serum or blood At 37 C the colonies from some strains are thin dry and irregular with rough edges In broth growth is diffuse at 22 C clumped masses and occasionally ring and pellicle formation are seen The organism does not liquefy gelatin Blood glucose and adequate aeration increase the yield of cultures Isolation of *P pseudo tuberculosis* from heavily contaminated material is facilitated by Endo Agar desoxy cholate citrate agar or the selective medium of Morris (1958)

Milk is not coagulated but is alkalinized slowly The following carbohydrates and alcohols are fermented without gas pro duction glucose maltose mannitol galac

* Revised with the assistance of Professor W Knapp Tubingen

petuate the infection, and their fleas may spread it to commensal rodents

The indispensability of the flea vector in the rodent flea cycle is well known Only a comparatively few fleas feeding on an infected rodent with severe bacteremia become infected and fewer still become infective The numbers of fleas that become infective are conditioned by species feeding habits whether zoophilic (as are many wild rodent fleas) or anthropophilic and the efficiency as a transmitter which varies with species and is greatly influenced by climate The longevity of fleas is very important for the carry over from one season to another The climate and the size of the rat population control the density of the flea population in urban situations density is measured by the flea index the average number of fleas on each trapped rat A cheopis index of at least 3 appears to prevail during epizootics The most important and efficient vector of rat plague throughout the world is *Xenopsylla cheopis* The common squirrel flea (*Diamanus montanus*) is not very efficient In wild rodent plague flea transmission from rodent to man apparently represents a weak link accounting for few transmissions to man Far more threatening is the introduction of plague by squirrel fleas into rat populations in rural and possibly urban areas (Meyer and Holdenried 1949)

When a person ill with bubonic plague develops plague pneumonia of metastatic origin he then may transmit the agent through droplets of sputum *P pestis* can be projected for several feet from the face of the patient when he coughs those exposed to this aerosol may then develop primary pneumonic plague This means of direct spread along with overcrowding in badly ventilated buildings low temperature and undesirable social habits and customs helps to bring plague to epidemic proportions (Meyer 1961)

Prevention and Control

Immunization against plague is too slow and unreliable for immediate prophylaxis Chemoprophylaxis of bubonic or pneumonic plague can be achieved with sulfonamides or antibiotics even though man cannot be made totally infection proof As long as the reservoirs remain untouched the threat persists Systematic warfare against rodents is the fundamental approach in an effective anti plague program The potent quick acting rodenticides—1 080 (sodium fluoroacetate) and antu (Pollitzer 1954 1960)—should be used by experienced persons to free cities of rats and to establish rodent free belts around towns and villages exposed to plague Zinc phosphide red squill and arsenious oxide are still popular Anticoagulants cause no bait shyness because they kill the rodents gradually and therefore can be used continuously until the animals have been exterminated (Steiniger 1956) As natural plague foci are affected by improvements in civilization and farming and as the methods for enzootic plague control are improved the elimination of enzootics may ultimately become practical (Fenyuk 1960)

Remarkable success against fleas has been achieved with residual insecticides DDT (5% in kaolin powder) dusted in and around houses supplemented by treatment of clothing bedding furniture rat runs and harborages has been invariably effective In fact a threatening epidemic of bubonic plague was aborted in Haifa with this insecticide alone

Treatment of epidemic plague victims can now be handled by mobile teams Therapy can be begun earlier, and patients need not be transported for long distances Physicians and nurses attending pneumonic or suspected pneumonic infections must wear hoods masks with goggles overalls and gloves Contacts and suspected contacts first are disinfected and segregated their temperatures are taken and chemoprophylaxis or chemotherapy is instituted The administration of sulfonamides (3 Gm per day for a week) appears to reduce the number of cases of pneumonic plague among those exposed The over all program for epidemic control requires the guidance of trained personnel—physicians entomologists mammalogists laboratory workers and sanitarians In this connection it is well to remember the words of Aubert Roche La civilization seule a detruit la peste en Europe seule elle l anéantira en Orient

In many countries the incidence of plague has decreased markedly within recent years

by intraperitoneal or subcutaneous injection feeding or inhalation guinea pigs rabbits sparrows and canaries are quite susceptible white rats usually are refractory to atoxic strains

The organism has been recovered from soil dust water fodder and milk (Schutze 1929)

Pathogenesis

The organism can gain entrance to susceptible animals through any of several different portals As a rule the abdominal viscera are primarily diseased Breaks in the skin can also serve as portals Young inadequately fed animals are more susceptible than well fed adults Guinea pigs in particular are very susceptible mice and rabbits less so Parenteral introduction of pure cultures is fatal to guinea pigs in 15 to 45 days at autopsy one finds local abscesses enlarged regional lymph nodes with caseous centers and white gray spots studding the spleen the liver the lungs and bone marrow When bacilli are ingested small necrotic nodules appear in the Peyer s patches of the ileum and the cecum, and there is caseous necrosis in the mesenteric lymph nodes and the omentum.

In the course of epizootics guinea pigs may exhibit 1 of 3 types of clinical manifestations septicemia fatal in 24 to 48 hours classic pseudotuberculosis in which there is emaciation diarrhea and death in from 3 to 4 weeks the glandular form with lesions of the cervical and the thoracic nodes probably transmitted through bites In any of these forms the bacillus may be in the blood stream Severe septicemia is usually rapidly fatal and at autopsy there are acute splenic tumor severe hemorrhagic enteritis and accumulation of clear fluid in serous cavities Characteristic are the whitish nodules in the liver the spleen and occasionally in the lungs They represent focal necrosis—coagulated cells granular debris and fragmenting polynuclear leukocytes many surrounded by foamy reticulum cells but rarely by epithelioid cells Giant cells are absent The necrotic center may contain blood vessels plugged with bacterial emboli (Knapp and Masshoff 1954) Chronic lesions show extensive fibroblast and epithelioid cell proliferations some of which become granulomatous but never calcify Bacilli are numerous in such lesions

Immunity

Immunization against this infection has been of interest because of the cross immunity with *P pestis* known long before the common somatic antigens were discovered Chloroform killed heat killed or formalin killed suspensions of *P pseudotuberculosis* protect guinea pigs and rats against infection with *P pestis* However the envelope antigen of the plague bacillus confers no immunity against *P pseudotuberculosis* and guinea pigs resistant to plague are still susceptible to pseudotuberculosis Antiplague sera usually agglutinate a variety of strains of *P pseudotuberculosis* to about the same titer Antipseudotuberculosis R sera react with *P pestis* strains (Thal 1956) *Antipseudotuberculosis or antiplague sera do not* confer passive immunity to guinea pigs

Guinea pigs that have recovered from the disease possibly remain latently infected and are immune to reinfection Active immunity may be produced in experimental animals through inoculation with avirulent living cultures (Thal 1954 1956 1962 Van Dorssen 1955 Sachdeva *et al* 1956) A solid immunity of over 5 months duration has been induced with the avirulent type IV strain (32) The resistance is anti infectious it protects against challenge with nontoxic strains and subtoxic doses of cultures of toxic strains but it does not protect against the exotoxin Antitoxic immunity can be produced with toxoid

Diagnosis

The disease in man cannot be distinguished clinically from typhoid paratyphoid tularemia cat scratch disease lymphogranuloma inguinale or certain viral infections *P pseudotuberculosis* is usually isolated on ordinary media without difficulty from the blood during life or from pathologic lesions at death Differentiation from *P pestis* in laboratory animals injected with plague-suspect material and from salmonella which produce no gas may be difficult Different media have been used but none is absolutely diagnostic With the selective medium

tose arabinose, glycerol isodulcitol, levulose rhamnose, trehalose, aesculin and xylose Acid is not formed in amygdalin dulcitol, erythritol inositol inulin, lactose, raffinose or saccharose Variations in fermentation of sorbitol salicin and dextrin, both at 22° and 37° C have been described (Thal 1954, Knapp 1959) *P pseudotuberculosis* breaks down urea, forms no indole and gives a positive methyl red and β galactosidase reaction (Mollaret and LeMinor 1962) Nitrates and methlyene blue are reduced rapidly A little H_2S is formed The catalase test is positive

Antigenic Structure

A multiplicity of different antigens has been recognized in *P pseudotuberculosis* namely 2 different thermolabile H antigens 5 type specific thermostabile O antigens defining 5 serologic types The subtypes IA IB IIA IIB IVA and IVB have been separated by absorption agglutination from types I, II and IV (Thal 1954 Knapp 1955 Girard and Chevalier 1955) At least 1 thermostabile antigen occurs in all types and seems to be very similar to an antigen present in *P pestis* (Schutze 1929 Kauffmann 1932 Thal 1954 Knapp 1960)

Extensive immunologic analysis has revealed that of the 16 antigens distinguishable in *P pestis* 13 are also found in *P pseudotuberculosis* (Bhagavan *et al* 1956)

Glycolipid antigens have also been isolated (Davies *et al*, 1958 Westphal *et al*, 1959)

Live cultures of avirulent strains of *P pseudotuberculosis* produce a solid immunity against virulent strains and also against *P pestis* in the guinea pig Lawton and Surgalla (1963) have isolated from an avirulent *P pseudotuberculosis* a protein lipopolysaccharide complex PF protective factor which can protect guinea pigs against plague as early as 1 day after vaccination But the relation of this PF antigen to plague and pseudotuberculosis immunity is still uncertain

On the other hand the antigenic relationship between *P pseudotuberculosis* type II and factors 4 and 27 of the salmonella B group and between *P pseudotuberculosis* type IV and factors 9 and 46 of the salmonella D group, lends support to the concept that the bacterium is related to the *Enterobacteriaceae* (Kaufmann 1932, Knapp, 1960)

Lysates and filtrates of certain toxic strains are lethal for mice guinea pigs rats and rabbits (Lazarus and Nozawa, 1948, Girard 1950 Thal 1954) The pathophysiologic effects of the pseudotuberculosis toxin are strikingly different from those of the murine plague toxin with respect to blood pressure changes hemoconcentration and local necrosis

A strain of *P pestis* phage lyses *P pseudotuberculosis* at 37° C This phage adapted to *P pseudotuberculosis* may lyse certain strains of Shiga Flexner, Sonne and Schmitz dysentery bacilli Claims that *P pestis* spontaneously transmutates to *P pseudotuberculosis* need more experimental confirmation

Resistance

Suspensions of *P pseudotuberculosis* in saline may survive 60° C for 3 hours but are inactivated within 5 to 10 minutes at 70° to 80° C One per cent phenol kills in 5 to 30 minutes, formalin 5 to 10 minutes 0 001 per cent mercury bichloride or silver nitrate in 120 minutes 60 per cent alcohol in ½ to 5 minutes (Knapp 1959) Drugs in the following concentrations stop multiplication neomycin 0 1 to 2 5 mcg/ml oxytetracycline chlortetracycline, tetracycline HCl, 0 25 to 2 5 chloramphenicol 0 5 to 6 25 streptomycin 1 25 to 10 penicillin 1 to 12 5, erythromycin 125 to 500 sulfonamides 12 5 to 60 mg (Knapp 1955) Lyophilized cultures or cultures on blood medium in sealed tubes remain viable for years

Distribution and Range of Pathogenicity

Pseudotuberculosis is a fairly common epizootic and enzootic disease in Europe sporadic infections have been observed in North and South America England Japan and India Infections among guinea pigs and turkeys are epizootic they are enzootic in rabbits and hares cats chickens pigeons swans canaries sparrows blackbirds monkeys sheep hogs horses lions foxes goats and doubtless other untested animals

The experimental disease can be produced

attention Masshoff and Dolle (1953) reported infection with the clinical symptoms of an acute or subacute appendicitis occasionally accompanied by enteritis associated with enlargement of the regional lymph nodes They described this disease as an abscess forming lymphadenitis Clinical and surgical findings were uniform An acute onset with temperatures of 100 to 104° F and pain in the middle or the right lower abdominal quadrant aroused the suspicion of appendicitis Laparotomy revealed a considerable amount of clear serous exudate and in most instances a normal looking appendix but the wall of the ileum and the cecum showed glassy rigid swelling due to infiltration In the mesentery particularly the ileocecal angle single enlarged lymph nodes or packets of enlarged lymph nodes were always present Knapp (1954) and Knapp and Masshoff (1954) identified this form of mesenteric lymphadenitis to be an infection with *P pseudotuberculosis* Since 1954 Knapp has fully proved the nature of the infection either through isolation of the bacterium or serologic tests and histologic investigations This form of pseudotuberculosis in children and young adults is by no means rare in Europe (Knapp 1963) Its course is usually benign with or without appendectomy In most cases the acute symptoms subside rapidly without complications or the use of antimicrobial therapy

Diagnosis is assured by isolation of the organisms from the lymph nodes feces (Daniels 1962) or blood and by microscopic examination of the nodes In the sera of acutely ill patients antibodies have been detected by means of the agglutination test with live smooth strains of types I to V of *P pseudotuberculosis* (Knapp 1956) Titers of 1 80 to 1 10 240 have been recorded Apparently the titer does not fall until the infection has been completely removed from the mesenteric nodes it may rise fall or remain stationary independently of clinical symptoms Complement fixing antibodies have been found less frequently and in lower titers (Knapp and Steuer 1956) Antibodies in most patients sera can be detected with living bacteria or bacteria killed by formalin or phenol but not with boiled bacteria

In order to determine the prevalence of pseudotuberculosis in man the proper serologic tests should be carried out in cases in which mesenteric lymphadenitis is suspected or other intestinal disorders are not readily identified If agglutination with type II or IV of *P pseudotuberculosis* occurs cross absorption tests with the related salmonella strains B and D are mandatory Recently Daniels (1961 1962) succeeded in isolating *P pseudotuberculosis* from the feces of a child and his canary

TULAREMIA

Francisella tularensis

Bacterium tularense (so named by McCoy and Chapin) causes a specific infectious disease of wild mammals and ancillary hosts in which it is maintained as a heterogeneous infection insects act as perennial reservoirs and vectors Man enters into the chain on contacting infected tissues or body fluids of certain mammals birds or insects Tularemia in man is an acute moderately severe febrile disease with a tendency to pneumonic complications the clinical picture varies considerably according to the mode of entry of the agent

The organism causing tularemia has been placed in several bacterial genera it is termed *Pasteurella tularensis* (McCoy and Chapin) in Bergey s *Manual of Determinative Bacteriology* (ed 7 1957) In a judicious study Philip and Owen (1961) recommended that it be considered as the type species *Francisella tularensis* (McCoy and Chapin) in the *Brucellaceae* This revision will be adopted in the next edition of Bergey s manual

History

Tularemia is not a new disease Its adaptation to rodent ectoparasites suggests that it is of considerable antiquity and has been probably long endemic in the Americas and Asia

In 1910 G W McCoy in his studies on plague discovered among ground squirrels a disease characterized by lesions similar to those of plague This disease was encountered in rodents shot or found dead in Tulare County California and McCoy and Chapin

developed by Morris (1958) *P pseudotuberculosis* can be isolated readily from the feces of rodents and man (Paterson and Cook 1963)

Freshly isolated strains exhibit the following differentiating characters (1) *P pseudotuberculosis* grows more rapidly and more luxuriantly than *P pestis* on artificial media (2) Its motility is always absent at 37° C and must be tested after repeated transplants at 22° to 30° C (3) It acidifies media containing rhamnose glycerine and melibiose (4) Urea is broken down by *P pseudotuberculosis* but not by *P pestis* (5) *P pestis* is virulent for the white rat, nontoxic *P pseudotuberculosis* is not (6) Polyvalent antiserum with antibodies against types I to V as a rule agglutinate *P pseudotuberculosis* but not *P pestis* According to Thal (1954) and Goyon (1956) type I is found most frequently types II and III occasionally and types IV and V rarely The sera of patients suffering from pseudotuberculosis may agglutinate bacilli isolated from the blood or the tissues in dilution of 1 80 to 1 10 240 (Knapp 1958) (7) A *P pestis* phage used in critical test dilutions at 20° C does not lyse *P pseudotuberculosis* (Gunnison *et al* 1951) The *P pseudotuberculosis* phages PST 87 102 103 and 105 (Girard 1943) lyse all strains of type I to V The use of this phage for the routine diagnosis of *P pseudotuberculosis* strains is recommended (Knapp 1962 1963) since it rapidly differentiates members of the *Pasteurella* group

Treatment

Little is known about the effect of antimicrobial drugs on pseudotuberculosis Two patients with the septicemic form who were treated with sulfonamides and antibiotics recovered (Snyder and Vogel 1943 Burianek *et al* 1949) 2 others died despite treatment (Hassig *et al* 1949) The benign enteral form requires no specific treatment recovery is uneventful (Knapp 1959 Daniels 1962)

Epidemiology Epizootiology and Control

The vast animal reservoir with its carriers and shedders is probably the source of most human infections Single sporadic cases of the septicemic form have been attributed to contact with cats and to ingestion of infected meat (Moss and Battle, 1941) or contaminated drinking water and food (Knapp 1958) Nothing is known about spread from one person to another

Sporadic and epizootic outbreaks during cold wet weather attest to the importance of predisposing factors Nothing definite is known about the portal of entry or the mode of transmission among animals, but the nearly constant involvement of the abdominal organs incriminates the oral route Fleas cannot transmit the infection Field mice deer otters and birds may serve as reservoirs (Borg and Thal 1961)

Pseudotuberculosis in Man

Until 1953 the medical literature contained only a few references to this infection in man (Hassig *et al* 1949, Knapp and Thal 1963) The clinical picture is dominated by a severe typhoidal course that ends fatally in many cases Vague prodromal malaise is followed by abrupt febrile onset with severe headache chills general pains anorexia and occasional catarrhal symptoms The fever of the irregular or septicemic type at times reaches 105° F Anorexia abdominal tenderness constipation or diarrhea and variable degrees of leukocytosis are usual Within a few days after onset the liver and the spleen become palpable and tender Other manifestations include septicemia, effusions into serous cavities, bronchitis pulmonary engorgement and edema and changes in the parenchyma of the liver the kidneys and the myocardium Death is usually preceded by icterus, toxemia and stupor it occurs usually between the 10th and the 24th day

Diagnosis may be established by cultures from blood or organ specimens and can be supported by a test for specific antibodies The characteristic findings at autopsy are nodular caseous or abscessed necrotic foci from 1 to 10 mm in diameter in the enlarged liver and spleen and occasionally the mesenteric lymph nodes and the pancreas

In contrast with the severe septicemic form the more frequent infections with a benign course in children and young people noted by Albrecht and Piechaud deserve

cytoplasm the growth pattern is influenced by the type and the amount of serum in the extracellular medium and by the nutrients in the medium (Merriott *et al* 1961)

Glucose maltose and mannose are fermented without gas production fermentation of glycerol levulose and dextrin is irregular (Francis 1942) The highly virulent American variety ferments glycerol All strains possess glutaminase and asparaginase only virulent strains degrade citrulline to CO NH_3 and ornithine (Marchette and Nicholes 1961) The cytochrome content is higher in virulent than avirulent strains (Mizuhara and Yamanaka 1961)

Antigenic Structure and Variation

Several strains studied by agglutination absorption tests seem to be antigenically uniform A serum prepared against *F tularensis* agglutinates *Brucella melitensis* and *Br abortus* to about 1/4 to 1/6 of the original titer (Francis and Evans) However neither organism absorbs the homologous agglutinins from the tularense serum Similarly *F Tularensis* is agglutinated to a low titer by antimelitensis and antiabortus sera but does not absorb the homologous agglutinins from these sera The heat labile antigen extracted from the plague bacillus does not precipitate with tularense antiserum but an extract of *F tularensis* does precipitate to a low titer with an antiplague serum (Larson *et al* 1951) There is no cross immunity with *P pestis* or *P pseudotuberculosis*

Ethyl ether releases from virulent or avirulent *F tularensis* an antigenic fraction not sedimentable by prolonged centrifugation at 4 500 rpm This ether soluble antigen is highly immunogenic in rats and mice (Larson, 1945a Bell *et al* 1952) Purification by salt precipitation differential centrifugation and dialysis yields a product of *uniform* immunogenicity Electron micrographs have shown that the product includes cell wall material

Ouchterlony diffusion tests of the ether extract have shown at least 4 and possibly 6 antigenic components the original aqueous supernatant of the extract contains 9 precipitation zones common to virulent and avirulent strains The carbohydrate content is high in the cell wall of low virulence strains (over 30%) but low (about 5%) in highly virulent strains (Guss *et al* 1962)

The ether soluble antigen consists of a polysaccharide and amino acid complex containing organic phosphorus Its serologic and immunogenic activity is very high 38 to 77 mcg is sufficient to immunize mice the degree of protection being proportional to the precipitation titer (Ormsbee *et al* 1955 Ormsbee and Larson 1955) Phenol extraction yields several crude antigenic fractions (Alexander 1950 Girard and Gallut 1951) acetone extraction of peptone broth cultures gives a protein or a protein-carbohydrate fraction (Downs *et al* 1947)

Virulent and avirulent strains can be differentiated by injection into mice guinea *pigs and rabbits of varying susceptibility and* by calculating the number of organisms of a given strain necessary to cause death (Owen *et al* 1955) Highly virulent strains in doses of 1 to 100 bacterial cells cause the death of all animals and numerous necrotic nodules are found in the spleen the liver and the lungs The less virulent strains are lethal to laboratory rabbits only in a dose of 10^9 bacilli

A relationship between virulence and immunogenicity has not been established conclusively As a rule cultures with an LD_{50} of 2×10^3 or more organisms in mice are not smooth the organisms multiply little or not at all in the tissues and are not immunogenic It is surmised that some as yet unidentified antigen may be responsible for the ability of a strain to multiply and to immunize (Moody and Downs 1955 Downs and Moody 1955 Moody 1955) Virulent and relatively avirulent strains do not differ significantly in yield of antigen or in immunogenicity (Owen *et al* 1955) The lower yield of antigen from the well studied avirulent *strain 38 is attributable to its slower* growth and the lower quantity of soluble cell wall antigens

Although live and killed vaccines produce agglutinins only virulent or the more immunogenic live vaccines stimulate precipitins detectable by agar diffusion technic (Tulis and Eigelsbach 1961)

Living virulent and avirulent strains injected intraperitoneally into mice in doses of 10^9 organisms cause death within less than

(1912) named the causative organism *Bacterium tularense* Wherry and Lamb diagnosed the first infection in man and recognized the hare to be an important source Francis in 1919 and 1920 investigated rabbit fever in Utah and discovered that the blood of an infected rancher bitten on the neck by a deer fly produced a plaguelike disease in guinea pigs In rapid succession Francis reported the isolation of *Bact tularense* from jack rabbits the transmission of the infection by bites of the deer fly and the rabbit louse, the cultivation of the organism on a new medium and the means for serodiagnosis

The importance of ticks as reservoirs and vectors has been elucidated by Parker Spencer and his co workers and by Calhoun Much of what is known about tularemia was learned through the extensive field laboratory and clinical investigations of Francis and his colleagues in the U S Public Health Service The cytotropism of the bacterium was first recognized by Francis and its significance was established by Buddingh and Womack (1941) Since 1926 murine enzootic and epizootic followed by water-borne outbreaks have assumed importance in Russia (Tigertt 1962) and the United States (Parker *et al* 1951) It has spread as an epizootic from West Siberia through Southeast Russia to the dry climate areas of Northern Central and Southern Europe where it is now an autochthonous disease (Jusatz, 1961) Antitularemia prophylaxis with live vaccine is used with success in the Soviet Union (Tigertt 1962)

Morphology

The shapes of *F tularensis* are diverse—large and small coccoid and bacillary oval minute filamented, bean shaped dumbbell shaped bizarre and so called involution forms Small units are filterable It has an extremely delicate structure of very low electron density which may account in part for its low survival rate when lyophilized (Shepard *et al* 1955) It has no capsules or flagella and is not motile (Hesselbrock and Foshay 1945) Dilute carbol fuchsin gentian violet or polychromatic eosinmethylene blue preparations will stain the bacterium in smears and sections

Cultivation and Biochemical Activities

F tularensis is rather fastidious in its growth requirements It grows readily on a semisolid medium such as gelatinized yolk of hens eggs (McCoy and Chapin) or on tryptose, thiamine cysteine sodium glycolate glucose and 5 per cent defibrinated rabbit blood agar (Gaspar *et al*, 1961) A chemically defined medium of 13 amino acids (high histidine content) spermidine phosphate thiamine, salts and glucose may yield from 18 to 55 $\times 10^9$ cells per ml after incubation for 16 to 24 hours at 37° C Strains of reduced virulence require the addition of calcium pantothenate or uracil adenine and guanine for maximal growth (Nagle *et al* 1960) Thiamine is an absolute requirement for all strains Blood may be replaced by plasma and catalase (Hood 1961) the clear peptic digest plasma is claimed to be superior for recovery of *F tularensis* from aerosols (Levin *et al* 1962)

The bacterium forms minute transparent droplike colonies that are mucoid and easily emulsified Variations in colony types are claimed to be associated with differences in virulence and immunogenicity Media inoculated with infective tissue may show discrete growth in from 2 to 7 days but in subcultures confluent growth appears in 24 to 48 hours Routine blood cultures may be made on Rhamy s hemoglobin-cystine agar or thioglycolate heart infusion agar Fully virulent cells grow luxuriantly in consecutive transfers on a variety of media if the inocula are large

For large scale cultivation liquid media composed of protein hydrolysates with extracts of blood cells are of value Decreased oxygen tension or large inocula are required to initiate growth Growth is optimum at 37° C and at pH 7 6

Strong buffers are essential to counteract the tendency of *F tularensis* to produce excessive ammonia from amino acids The bacterium finds a suitable environment within cells either of mammals or of embryonated egg (Downs *et al* 1947) Multiplication of *F tularensis* in the cytoplasm of the H strain of mouse fibroblast maintained in Scherer s solution containing 10 per cent horse serum causes slight changes in the

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2 days however killed organisms are not toxic (Moody and Downs 1955) It has been reported that *F tularensis* yields both a toxic filtrate and an endotoxin Suspensions of formalized bacteria contain a heat stabile antigenic fraction unrelated to virulence that causes characteristic dermal reactions in rabbits, this fraction can be neutralized with antiserum and its effect is partially inhibited by previous vaccination (Larson 1946) Treatment with ether removes this dermo necrotic activity Lipopolysaccharides isolated from virulent or live vaccine strains are nontoxic but stimulate agglutinin pro duction (Stefanye 1961)

Resistance of the Organism

This organism has survived in cultures for 22 years at 10° C without transfer in car casses for 133 days in hides for 40 days in water for up to 3 months in pure glycerin at −14° C for 2 years and at −70° C for months Virulent organisms have survived in the muscle of refrigerated infected rabbit carcasses for 4 months Dried in vacuo and held at room temperature it remains viable for several years Heating of cultures at from 55° to 60° C for 10 minutes kills the organism tricresol (1%) 0 1 per cent for malin and 1 0 ppm of chlorine inactivate it 0 5 mg per ml of methylene blue is in hibitive in vitro but not in vivo (Yaniv and Avi Dor 1952) The bacteriostatic level against 10^6 cells per ml in a suitable medium is 2 5 mcg per ml for either chlortetracy cline or chloramphenicol

Range of Parasitism

Burroughs and his co workers (1945) listed 48 different species found infected with tularemia including cottontails jack rabbits and snowshoe rabbits In North America the cottontail rabbit is the source of over 70 per cent of all human cases of tularemia (Jellison and Parker 1945) Other animal sources are the gray squirrel fox squirrel opossum woodchuck muskrat skunk, coyote fox cat sheep deer vole and bull snake Several epizootics have occurred among field mice in cultivated areas of Nevada Oregon and northern California contamination by mice of streams wells and mud during the winter favors the spread of the infection (Kartman *et al* 1959) Epizootics among muskrats have contaminated mud and stagnant water (Fyvie *et al* 1959) Epizootics among con tinental voles and water rats have caused explosive outbreaks in southern Russia fol lowed by westward invasion of tularemia into middle and western Europe Beginning in 1948 field mice then rabbits hares, ham sters and lemmings underwent mass mortali ties creating firmly established foci in the indigenous fauna of central Europe (Gel man 1961 Jusatz, 1961) Squirrels chip-munks calves and dogs have been found to be naturally infected but so far have not been known to cause human disease

Game birds and domestic chickens are among the avian sources The organism has been isolated from two species of grouse the sage hen and the horned owl but human infections have not been traced to them

Epizootic tularemia in pen raised beavers was conveyed by the water supply from a drainage ditch later found to be contami nated (Bell *et al* 1962)

Certain species of ticks are important ar thropod reservoirs (Gelman 1961) Survival in at least 54 arthropods has been reported The ticks known to be capable of transmit ting the infection to man are the Rocky Mountain and the western wood ticks the common eastern dog tick the Lone Star tick one species of deer fly and the mosquito

F tularensis has been isolated from vari ous rabbit and other rodent fleas but they do not transmit the disease to man Attempts to infect fleas by feeding them on infected hosts were largely successful however the fleas did not transmit the infection to mice or guinea pigs (Parker and Johnson 1957) Body lice and their feces kept at low humid ity and temperature have remained infected for 53 days (Price 1954)

During the period from 1931 to 1940 in ecologic studies in the U S S R the frequent occurrence of outbreaks in man of tularemia was proved to be due to use of water con taminated during rodent epizootics

Pathogenicity and Pathogenesis

In man *F tularensis* can be recovered during the first week of disease by inoculat ing guinea pigs or by direct culture on suit able media Pus taken from suppurating

lymph nodes early in the disease contains viable bacteria but later the lymph nodes do not Difficulties in isolating *F tularensis* are probably associated with the small number of bacteria in the tissues and their low viability At autopsy in man and animals the acute necrotic lesions in the liver the spleen the lungs and bone marrow readily yield positive cultures Inoculation of guinea pigs with tissues from field mice occasionally has revealed the infective agent when latent disease was not anatomically visible (Burroughs *et al* 1945)

Susceptibility appears to be in decreasing order mice hamsters guinea pigs rabbits rats monkeys dogs and 9 day old chicks Rats are 1 000 times more resistant than white mice Even cold blooded animals (e g reptiles and catfish) are susceptible The sheep is the only large animal in the United States in which tularemia has occurred in true epizootics it can serve as an important source of infection in humans (Jellison and Kohls 1955)

The histogenesis of the typical lesions is characterized by a rapid accumulation of mononuclear wandering cells principally macrophages polymorphonuclear cells play little part in the reaction The reaction may lead to necrosis or to granuloma formation Vascular changes though conspicuous in advanced lesions are not responsible for the necrosis nor is anoxemia (Lillie and Francis 1936) Of significance are the capacity of *F tularensis* to multiply within hepatic and endothelial cells of guinea pigs and its selective affinity for thin ectodermal cells (Budding and Womack 1941)

F tularensis is a facultative intracellular parasite that may persist for years in the organs (Foshay and Mayer 1936 Carr and Kadull 1957) According to Francis (1937) man can be infected with *F tularensis* in at least 20 ways The most important are contact with infected vertebrates and discharges of arthropods bites of mammals (particularly carnivores) or arthropods ingestion of infected water or partially cooked infected meat of vertebrates inhalation of air containing material from cultures infected laboratory animals and fecal droplets of ticks When the bacteria penetrate the skin or the mucosa in about 10 per cent of the human infections a papular primary lesion develops and soon ulcerates The lesion of the ulceroglandular form of tularemia usually appears on the hands the arms the face or in the conjunctiva In some patients the disease takes the septicemic form at onset and may cause death in 4 to 12 days

From the site of inoculation the bacteria spread along the superficial and the deep lymphatics leading to dermal lymphangitic nodules lymphadenitis and bubo formation in more than 90 per cent of human infections In the absence of discoverable primary lesions lymphangitic invasion is rare The bacteremia gives rise to diffusely scattered foci of necrosis in the spleen the liver the lungs lymph nodes bone marrow and possibly other tissues and organs With the appearance of antibodies bacteremia disappears but new lesions may develop by lymphatic extension A second and invariably fatal invasion may occur which usually disperses bacteria through both the systemic and the pulmonary circulatory systems resulting in miliary and submiliary necrosis in nearly every organ

Ingestion of infected animal tissue or water giving rise to the enteric form of tularemia causes local reactions necrotizing pharyngitis abscesses in the roof of the mouth enlargement of the submaxillary and the cervical lymph nodes and ulcers hemorrhages and minute necrotic lesions in the gastrointestinal tract The resulting infection and febrile disease resembles typhoid fever with gastrointestinal signs and toxemia It is likely to be fulminant and fatal unless recognized and treated properly

Pulmonary involvement in tularemia is extremely common The incidence of tularemia pneumonia without ulceroglandular lesions is much greater than is generally thought Parenchymal involvement with or without enlargement of the hilar lymph nodes or pleural effusion occurs Confluent lobular pneumonia similar to caseous tuberculous pneumonia followed by gangrene and abscess formation has been reported (Ivie 1955) Primary tularemic pneumonia also occurs The high incidence of pneumonia in what appears clinically to be typhoidal tularemia suggests that the organism frequently gains access to the body through the respira

tory tract Man can be infected readily by as few as 25 organisms when exposed to an aerosol composed of 1-μ particles (McCrumb 1961)

Following ocular infection, the typical primary lesion is an ulcerated papule on the lower eyelid followed by general infection of the conjunctival sac characterized by congestion of the vessels lacrimation damage to the eye and involvement of the lymphatics

IMMUNITY

An attack of tularemia confers a relative immunity Butchers having suffered one attack are not known to experience others despite probable frequent exposure Reinfection in laboratory workers produces illnesses of varying severity from small local lesions and no constitutional reactions to acute febrile illness followed by mild prolonged systemic complaints (Green and Eigelsbach 1950)

The in vitro bactericidal activity of normal plasma is highest in the cow the dog and the sheep lower in the guinea pig the monkey the human the rabbit the rat and the mouse This correlates to some degree with susceptibility to experimental infection (Stanziale 1957)

Heat or formalin killed vaccines provoke severe local and constitutional reactions Vaccines detoxified with sodium nitrite and acetic acid (Foshay) administered annually do not confer complete resistance but modify favorably the course of the disease Similar results have been obtained with phenolized or acetone extracted vaccines (Kadull *et al* 1950)

Considerable resistance has been produced in rabbits and rats (Larson 1945a Downs *et al* 1947) with killed organisms or lysates but the same vaccines protect only a small proportion of mice against as few as 2 to 5 LD_{50} of organisms The two vaccines that completely protect 40-day old mice are the ether-extracted vaccine of Larson and the purified antigen preparation derived from the cell wall (Larson *et al* 1954 Ormsbee and Larson 1955) It is believed that the dosage and the virulence of the challenge strain (425) are much higher than that ordinarily met under natural conditions in human infections In the rodent disease the mortality rate is nearly 100 per cent, whereas in the human disease the rate is 7 5 per cent in untreated cases

Early claims of Soviet and Turkish workers that live strains of low virulence were superior to a killed virulent culture for vaccination of guinea pigs are now supported by American studies and have been extended to mice and man An attenuated hypovirulent strain of *F tularensis* designated LVS (live vaccine strain), selected from a Russian vaccine and brought to the United States in 1956 has been used in animals and man (Eigelsbach and Downs 1961) Inoculation of 100 000 viable cells of this strain intracutaneously or 270 000 aerogenically into monkeys leads to multiplication locally and in the regional lymph nodes the liver and the spleen Antitularense gamma globulin appears in the plasma cells of regional nodes as early as the 3rd day after infection (McGavran *et al*, 1962)

The first human trial with a living avirulent strain administered intradermally induced local and general reactions in 72 per cent, with prolonged moderate disability in some and fairly severe disability in a few (Kosmachevskiy, 1944) In a more recent test lyophilized vaccine was applied cutaneously by scarification to exposed groups—slaughterhouse and agricultural workers There were local skin reactions accompanied by enlargement of the regional lymph nodes systemic reactions were relatively few, allergic reactions developed in persons who had had tularemia or had been immunized successfully The protection was reported to be effective in 90 to 96 per cent of cases and lasted for at least 6 years The use of avirulent living vaccine is now part of tularemia control in the Soviet Union (Tigertt 1962) In tests in human volunteers in the United States the living attenuated vaccine protected 10 of 14 whereas the cell wall antigen of Larson or the Foshay killed vaccine protected few or none against intracutaneous or respiratory challenge (McCrumb 1961 Saslaw *et al* 1961)

Agglutination or hemagglutination titers or immunodiffusion precipitin lines in prechallenge sera did not permit a prediction of whether or not an individual would become ill after challenge (Saslaw *et al* 1962)

Available evidence suggests that acquired immunity to virulent *F tularensis* develops only after natural infection or vaccination with hypovirulent strains Immunity produced by immunization with nonviable antigens protects only against strains of less than full virulence

Allen (1962) succeeded in passively transferring resistance to fully virulent strains of *F tularensis* to normal mice by viable spleen cells or peritoneal leukocytes from donors recovered from infection with an attenuated strain of *F tularensis* The degree of passively transferred resistance depended on the number of viable immune cells and the resistance persisted only as long as the transferred tissues were compatible with the tissues of the recipients These results and those of Stansberry and Woodward (1962) in rats support the hypothesis that immunity to fully virulent strains is associated in some way with an altered state of the tissues

It is generally agreed that a tuberculin type hypersensitivity follows previous exposure to *F tularensis* and therefore constitutes a reliable test for the diagnosis of abortive or asymptomatic forms of the infection (Ljung 1958) In animals the delayed hypersensitive and the immune responses are independent manifestations and a tularin reaction does not always imply immunity (Gordon 1963)

Diagnosis

When a patient has killed skinned and cleaned rabbits or has been bitten by deer flies other arthropods or by a mammal in an endemic area tularemia should be suspected In many cases however the mode of infection is obscure Clinical findings that may suggest the diagnosis include a febrile influenzalike attack with initial severe fever a temporary remission and a further febrile bout of 2 weeks duration followed shortly by a local lesion possibly conjunctivitis and tender enlarged lymph nodes The pneumonic type particularly difficult to diagnose clinically is an atypical pneumonia A correct diagnosis is usually made by a combination of clinical epidemiologic bacteriologic and serologic methods Contrary to views that *F tularensis* is rarely recovered it can be isolated from sputum pharyngeal or gastric washings with relative ease when the use of guinea pigs and the newer artificial media are combined (Larson 1945 Overholt *et al* 1961) The ultimate identification is made by specific staining using the fluorescent antibody technic with high titer sera produced in fowl and absorbed with *Pseudomonas* to eliminate cross reactions (Yager *et al* 1960)

Specific diagnosis has centered around the agglutination reaction Agglutinins appear not before the 7th day and sometimes not until late in the 3rd week of the illness a 4 fold rise is observed on the average on the 19th day of illness and the mean peak titer is reached some time during the 2nd and the 3rd months of the disease Thereafter the agglutinins decline slowly but they may remain detectable as long as 28 years or perhaps for life (Pullen and Stuart 1945) Agglutinins elicited by vaccination behave similarly to those in natural infection if a vaccinee contracts tularemia the rise of the agglutinins is no more prompt than in a nonvaccinated person who acquired the infection Cross reactions with *Br abortus* immune sera occur but do not cause diagnostic difficulties

The hemagglutination test using type O human red cells sensitized with tularensis polysaccharide has been used in an attempt to obtain earlier results (Wright and Feinberg 1952) Hemagglutinins reach diagnostic levels on the 11th day (according to Knothe and Havemeister 1961 on the 5th day) and reach a mean peak titer of 1 10 240 during the 2nd month they persist in the serum for years at somewhat higher levels than agglutinins Hemagglutination titers rise more rapidly than agglutination titers in tularemia just as they do in plague and salmonellosis (Charkes 1959)

Foshay (1950) reported 2 types of intradermal tests—one with killed bacteria the other with hyperimmune goat serum Positive reactions to both tests during the 1st week of illness were given by 99 of 108 patients and the other 9 became positive during the succeeding week The tests with killed bacteria have the disadvantage of requiring 48 hours before they could be read whereas hyperimmune goat serum gives an immediate wheal and flare To date this is

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Considerable resistance has been produced in rabbits and rats (Larson 1945a Downs *et al* 1947) with killed organisms or lysates but the same vaccines protect only a small proportion of mice against as few as 2 to 5 LD_{50} of organisms The two vaccines that completely protect 40 day old mice are the ether-extracted vaccine of Larson and the purified antigen preparation derived from the cell wall (Larson *et al* 1954 Ormsbee and Larson 1955) It is believed that the dosage and the virulence of the challenge strain (425) are much higher than that ordinarily met under natural conditions in human infections In the rodent disease the mortality rate is nearly 100 per cent whereas in the human disease the rate is 7 5 per cent in untreated cases

Early claims of Soviet and Turkish workers that live strains of low virulence were superior to a killed virulent culture for vaccination of guinea pigs are now supported by American studies and have been extended to mice and man An attenuated hypovirulent strain of *F tularensis* designated LVS (live vaccine strain), selected from a Russian vaccine and brought to the United States in 1956 has been used in animals and man (Eigelsbach and Downs 1961) Inoculation of 100 000 viable cells of this strain intracutaneously or 270 000 aerogenically into monkeys leads to multiplication locally and in the regional lymph nodes the liver and the spleen Antitularense gamma globulin appears in the plasma cells of regional nodes as early as the 3rd day after infection (McGavran *et al*, 1962)

The first human trial with a living avirulent strain administered intradermally induced local and general reactions in 72 per cent with prolonged moderate disability in some and fairly severe disability in a few (Kosmachevskiy 1944) In a more recent test lyophilized vaccine was applied cutaneously by scarification to exposed groups—slaughterhouse and agricultural workers There were local skin reactions accompanied by enlargement of the regional lymph nodes systemic reactions were relatively few allergic reactions developed in persons who had had tularemia or had been immunized successfully The protection was reported to be effective in 90 to 96 per cent of cases and lasted for at least 6 years The use of avirulent living vaccine is now part of tularemia control in the Soviet Union (Tigertt 1962) In tests in human volunteers in the United States the living attenuated vaccine protected 10 of 14 whereas the cell wall antigen of Larson or the Foshay killed vaccine protected few or none against intracutaneous or respiratory challenge (McCrumb 1961 Saslaw *et al* 1961)

Agglutination or hemagglutination titers or immunodiffusion precipitin lines in prechallenge sera did not permit a prediction of whether or not an individual would become ill after challenge (Saslaw *et al* 1962)

Men and women of all races and all ages are susceptible The incidence reflects exposure In the United States the highest annual incidence on record is 261 cases in 1939 The mortality rate in 15 525 cases reported from 1915 to 1942 was 6 9 per cent (Francis) The lowering of the death rate in recent years is attributable to the use of streptomycin However the mortality rate in one series of 53 cases of typhoidal tularemia was 19 per cent despite the use of antimicrobial drugs

The key factors in the transmission are ticks lice and mites which keep the infection alive in vertebrates For its propagation in nature *F tularensis* depends on the wood tick and the dog tick which feed on rabbits and other rodents The Lone Star tick together with the dog tick are the common reservoir and vector (Calhoun 1954) Of the many animals found infected in nature probably only a few serve as key reservoirs in any environment Human disease usually does not take place unless at least 1 per cent of the rodents in the region are infected The chain of infection is efficiently maintained ticks in all stages from larvae to adults are transmitters adult females pass it to succeeding generations transovarially The multiple factors affecting the bionomics and the ecology of the insect and the rodent reservoirs vary from region to region and from country to country creating a complex ecologic picture that has been sketched only in its broadest outlines Importing wild rabbits from regions of known endemicity into a tick ridden area creates new geographic reservoirs as experienced in Massachusetts (Ayres and Feemster 1948)

Control

Endemic tularemia of rodents cannot be eradicated An increased incidence in human beings always coincides with an increase in the infected rodent reservoir Under certain circumstances supervised field rodent poisoning campaigns are indicated Incidence probably would be reduced somewhat if interstate shipments of wild hares and their sale for food in markets and restaurants were supervised Sportsmen butchers and those who live in regions where the infection prevails must be educated to the dangers of this disease To render meat of rabbits harmless thorough cooking is necessary Rubber gloves should be worn while dressing rabbits Drinking water from streams in endemic regions should be avoided Laboratory workers should be protected by vaccination face masks and rubber gloves Isolation of patients is not necessary but discharges from suppurating local lesions must be disinfected

While in the past the tularemia enzootics and epizootics in the Soviet Union invariably led to manifestation of the disease in man it has now become possible to prevent such infections by prophylactic measures in agricultural practices and by poisoning campaigns directed against rodents as well as through wholesale antitularemia vaccination with living strains (Olsufjev and Rudnev 1960)

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the only method of making a positive diagnosis on the 1st day of illness Unfortunately it is difficult to obtain normal commercial goat serum for control and the use of the test in vaccinated persons is limited by the persistently positive reaction obtained up to 1 year after vaccination (Foshay and Mayer 1936 Friedewald and Hunt 1939)

An accelerated slide agglutination test which makes use of the reaction of tularemia antiserum with an antigen prepared from the spleen of a rodent suspected dead of tularemia (Vereninova *et al* 1962) is probably a modification of the Ascoli thermoprecipitin test developed by Larson (1951) and used very successfully in field surveys (Bell *et al* 1959 McDowell *et al* 1964)

Treatment

Streptomycin is bactericidal and is rapidly curative if administered in daily doses of 0 5 to 1 Gm It should be given for 5 to 8 days The broad spectrum antimicrobial drugs are also effective but less so than streptomycin The sensitivity of *F tularensis* to erythromycin varies greatly (Biegeleisen and Moody 1960) The tetracyclines or chloramphenicol given orally in an initial dose of 25 mg/Kg of body weight and then in doses of 0 5 to 0 75 Gm every 6 to 8 hours are also recommended (Ransmeier *et al* 1949) The effect of the drugs is dramatic in acutely ill patients treated early Strains resistant to streptomycin but not to chloramphenicol or tetracycline have been encountered

Epidemiology

Tularemia has been reported from many parts of the United States There were from 11 to 25 cases per 100 000 population in Arkansas during the period from 1937 to 1951 In 5 New England states only 20 cases have been observed since the discovery of the disease and in Vermont none at all In the United States between 1924 and 1950 there were 25 294 cases (Olsufjev and Rudnev, 1960) and between 1944 and 1955 the United States Public Health Service received reports of 10 865 cases Contact with cottontail rabbits was responsible for 65 5 per cent (Jellison and Parker 1945) In California 81 per cent were attributed to contact with wild jack rabbits (Simons *et al* 1953) Tularemia has been reported from Alaska (Philip *et al* 1962), Canada Venezuela Mexico and Japan and since 1926 epizootic waves have spread from Siberia to Turkey Iran and Israel and over most of Europe Cases have been reported from Africa (Gelman 1961)

It is an essentially sporadic disease It may become epidemic when a number of persons take an infected meal (Amoss and Sprunt 1936) when contaminated drinking water is consumed (Schmidt 1947 Parker *et al* 1951) or when deer flies are particularly infective (Hillman and Morgan 1937) No other infection has such a variety of modes of transmission but human to-human infection has not been recorded Numerous reports re-emphasize the well known risk of laboratory infection (Overholt *et al* 1961)

The most common clinical ulceroglandular or glandular types may be attributable to direct contact with infected animals for example in skinning or eviscerating through a break in the skin or through conveyance of the organism into the conjunctival sac Intermediate contact through infected blood sucking arthropods chiefly ticks biting flies and even mosquitoes may cause any of the 4 clinical types In typhoidal tularemia contracted by heavily exposed laboratory workers it is usually difficult or impossible to determine the portal of entry The virulence of the invading strain may partly determine which clinical type will develop

Seasonal occurrence with its peaks in summer is associated with rabbit hunting and arthropod vector activity in the Western United States Ticks are particularly dangerous from March to August deer flies from June to September Jack rabbits are hunted particularly from April to October and are a threat at the time In the East cottontail rabbits are hunted from November to January the months during which the human infections occur The periodic floods in the Don Delta of the USSR favor migration concentration and mixing of the populations of water voles which facilitate epizootics and tularemia in human beings far from the usual habitat of the rodents Prophylactic measures are thus greatly handicapped (Luk yanchenko *et al* 1961)

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TABLE 1 MAJOR SPECIES OF BRUCELLA

SPECIES	CO REQUIREMENT	H₂S REQUIREMENT	GROWTH ON THIONIN	GROWTH ON BASIC FUCHSIN	AGGLUTINATION IN MONOSPECIFIC ABORTUS SERA	AGGLUTINATION IN MONOSPECIFIC MELITENSIS SERA
Br melitensis	−	−	+	+	−	+
Br abortus	+	+ 4 days	−	+	+	−
Br suis	−	++ 5 days	+	−	+	−

tween the abortus and the melitensis organisms were found by Evans (1918 1923) during studies of pathogenic bacteria in milk Feusier and Meyer (1920) recommended that the three species be placed in a newly created genus *Brucella* because of similarities in their bacteriologic and serologic features and similarities in the infections produced by them in guinea pigs and monkeys

After a period marked by extreme controversy infectivity of *Br abortus* for man was accepted due in large measure to the identification of *Br abortus* in milk known to have been ingested by patients and to the identification of *Br abortus* as the etiologic agent of undulant fever in Rhodesia as well as of a similar case in the United States (Orpen 1924) *Br abortus* differs from the other species in its reduced ability to cause disease in man Epidemiologic and clinical data point to the fact that large numbers of *Br abortus* may be ingested over a long period of time via contaminated milk without producing severe illness in many of those exposed Under other circumstances e pecially via other routes of infection *Br abortus* may cause severe and even fatal infections

An agent for vaccination against bovine brucellosis became available through the isolation of an attenuated strain of *Br abortus* by Buck in 1923 Designated as *strain 19* it is so attenuated as not to be excreted in milk from vaccinated animals Despite its safety in cattle it is still capable of causing disease in other animals including man under certain conditions

CLASSIFICATION OF BRUCELLA SPECIES

The tests used most widely for classification of brucella strains are

1 Requirement for increased CO concentration in the environment a quality which carries weight especially when used to study cells obtained on primary isolation from the host

2 The production of H S for a continuous period of 4 to 5 days

3 Bacteriostatic action of basic fuchsin and thionin incorporated in solid media and

4 Agglutination in monospecific sera This allows separation of *Br abortus* and *Br suis* from *Br melitensis*

The differences between the species are quantitative and no single test suffices for certain identification

With the four tests the major biotypes or species segregate themselves as shown in Table 1

Strains deviating from this standard pattern may also show an unusual geographic incidence (Cruickshank and Madge 1954 Pickett and Nelson 1955) The fermentative and oxidative metabolism of the various brucella biotypes is also used for classification (Meyer and Cameron 1961 Meyer 1961) For example some cultures behave biochemically and serologically like *Br melitensis* (Great Britain Germany Uganda) but react oxidatively like *Br abortus* and strains that react serologically like *Br abortus* show the oxidative pattern typical of *Br melitensis* (Wundt 1962)

The earliest suggestion of the existence of bacteriophages for brucella species was given by the work of Pickett and Nelson (1950 1951) The actual isolation of brucellaphages in the U S S R by Popkhadze and Abashidze (1955) and by Parnas in Poland contributed a reagent active against *Br abortus* and when used in much larger amounts also active against *Br suis* Comprehensive studies have been made of the lytic action of the existing phages against

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28

The Brucellae

INTRODUCTION

The brucellae are aerobic small, gram negative coccobacilli which are nonmotile and nonsporulating Capsules when present are small Some strains require added carbon dioxide for growth on laboratory media The organisms grow poorly on ordinary media and growth does not occur under strictly anaerobic conditions There is little fermentation of carbohydrates in usual media oxidative action on various substrates is of value in classification Urea is hydrolyzed to a variable extent and catalase activity is high The organisms are classified on the basis of dye tolerance H S production CO requirement, agglutination in monospecific serum oxidation of glutamic acid ornithine ribose and lysine and susceptibility to specific bacteriophage They are characterized by an intracellular existence in the host In animals such as cattle sheep goats and pigs genital and mammary gland infection is a common event and placentitis occurs with premature expulsion of the fetus. Brucellosis in man produces a wide range of illnesses

Many names have been used to describe various forms of the disease undulant fever Mediterranean gastric remittent fever Neapolitan disease Malta fever Texas fever Rio Grande fever and Bang s disease In animals brucellosis is also known as Bang s disease, infectious abortion or epizootic abortion

HISTORY

Precise information concerning brucellosis began with the description of the clinical signs by Marston (1861), writing on gastric intermittent fever *Brucella melitensis* type species of the genus and the etiologic agent of the disease as it occurred on Malta was isolated from the spleen of a human case at necropsy by Bruce (1887) who named the organism *Micrococcus melitensis* Because of the high incidence of this disease in military and naval personnel stationed on Malta the British government created the Mediterranean Fever Commission in 1904 the responsibility of which was to determine the source of the disease and means for its control The work of this group was rewarded by the isolation of the organism from milk and urine of apparently healthy goats following the detection of brucellar agglutinins in their sera (Zammit 1905) The source of human Malta fever was thus clarified at once and when the consumption of raw goat s milk was stopped the incidence of the disease among military and naval personnel declined markedly

Brucella abortus was isolated by Bang (1897) from cases of bovine infection and a third species *Brucella suis* was isolated by Traum (1914) from the fetus of a sow (see Hayes and Traum 1920)

The three organisms were regarded as members of distinct genera until the close bacteriologic and serologic relationships be

TABLE 1 MAJOR SPECIES OF BRUCELLA

			Growth		Agglutination in Monospecific	
Species	CO Requirement	H S Requirement	On Thionin	On Basic Fuchsin	Abortus Sera	Melitensis Sera
Br melitensis	–	–	+	+	–	+
Br abortus	+	+ 4 days	–	+	+	–
Br suis	–	++ 5 days	+	–	+	–

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the brucella biotypes (Brinley Morgan, 1962 cf Parnas 1963) The limited range of lytic activity of the brucellaphages limits their suitability for use in classification and epidemiologic studies (Jones 1960, Jablonski 1962 Van Drimmelen 1960)

The combined use of classic tests, oxidative metabolic rate analyses and phage susceptibilities has led to a provisional description of the major species of brucella as follows

Brucella melitensis Aerobic Produce no H_2S or no more than a trace of H_2S on ordinary media Usually grow in the presence of basic fuchsin and thionin M antigen usually predominant Oxidize L alanine D alanine, L asparagine and L glutamic acid Do not oxidize L arabinose D galactose D ribose D xylose, L arginine DL citrulline DL ornithine or L lysine Not lysed by brucella phage Tbilisi (Tb) at routine test dilution Usually pathogenic for goats and sheep but can also affect other species including cattle and man

Brucella abortus Usually require added CO_2 for growth especially on primary isolation Usually produce moderate amounts of H_2S but may be negative Usually grow *in presence of basic fuchsin but inhibited by* thionin A antigen usually predominant Oxidize L alanine, D alanine L asparagine L glutamic acid L arabinose D galactose and D ribose do not oxidize D xylose L arginine DL-citrulline DL-ornithine or L lysine Cultures in the smooth or smooth intermediate phase are lysed by brucella phage Tb at routine test dilution Usually *pathogenic for cattle causing abortion* but can also affect other species including man

Brucella suis Aerobic Produce large amounts of H_2S or none at all Grow in the presence of thionin but usually inhibited by basic fuchsin A antigen usually predominant Oxidize L alanine D alanine L glutamic acid L arabinose D galactose D ribose D xylose L-arginine DL citrulline *DL-ornithine* and L lysine Do not oxidize L asparagine Not lysed by brucella phage Tb at routine test dilution Usually pathogenic for pigs but can also affect hares and other species including man

The provisional biotypes are described in Table 2

New biotypes given the designation *Br neotomae* and *Br ovis* are still under discussion *Br neotomae* isolated from the desert wood rat resembles *Br suis* and *Br abortus* but retains distinctive dye sensitivities and the ability to ferment xylose arabinose, glucose, levulose and galactose This fermentative activity is not shared by existing accepted brucella biotypes (Stoenner and Lackman 1957)

Br ovis the etiologic agent of ram epididymitis (Simmons and Hall 1953 Buddle 1953), has been observed only in the nonsmooth colonial phase and is not agglutinated by sera prepared against smooth brucella strains Antisera prepared against a suspension of *Br melitensis* containing smooth and nonsmooth colonial phases agglutinate the ovine strains as well as suspensions of rough or mucoid cells of *Br arbortus Br ovis* ferments glucose maltose mannose and trehalose in Pickett and Nelson s method (1955) It is of considerable interest that ram epididymitis is strikingly controlled in nature by immunization with strain Rev I of *Br melitensis* (Van Drimmelen 1960 Van Heerden and Rensburg 1962)

Brucella strains isolated from northeastern Siberian reindeer have been described as a new species *Br rangiferis* (Pinigin and Petukhova, 1962 Cherchenko and Bakaeva 1962) although the findings suggest that it should be included in the *Br melitensis* group (Reviews on the classification problems are found in Renoux *et al* 1955 Wundt 1961 Biberstein and Cameron 1961 the latter providing *critical appraisal of the entire family Brucellaceae*)

MORPHOLOGY AND PHYSIOLOGY

The pleomorphism of these very small bacteria is a function of the age of the culture and the environmental conditions in the medium Although the organisms are typically rod shaped very short rods with pointed ends coccal forms and coccobacillary forms may predominate In an analysis of more than 300 strains it was shown that only 6 per cent of the *melitensis* strains and 46 per cent of the *abortus* strains were predominantly rod shaped The coccal forms of *melitensis* predominate in primary cultures

TABLE 2 DIFFERENTIAL CHARACTERS OF THE THREE SPECIES OF THE GENUS *Brucella* AND THEIR BIOTYPES[1]

Species	Type	CO Requirement 5–10	H S Production	Growth on Dyes* Thionin a	Thionin b	Thionin c	Basic Fuchsin b	Basic Fuchsin c	Agglutination in Monospecific Sera A	M	Phage Tb at RTD	Metabolic Tests† Glutamic Acid	Ornithine	Ribose	Lysine	Most Common Host Reservoir	Køster Stain	Stamp Stain
Br melitensis	1	−	−	−	+	+	+	+	−	+	−	+	−	−	−	Sheep goat	Red	Red
	2	−	−	−	+	+	+	+	+	−	−	+	−	−	−	Sheep goat		
	3	−	−	−	+	+	+	+	+	+	−	+	−	−	−	Sheep goat		
Br abortus	1	+(−)‡	+	−	−	−	+	+	+	−	+	+	−	+	−	Cattle	Red	Red
	2	+	+	−	−	−	−	−	+	−	+	+	−	+	−	Cattle		
	3	−(+)	+	−	−	+	+	+	+	−	+	+	−	+	−	Cattle		
	4	+(−)	+	−	−	−	+	+	−	+	+	+	−	+	−	Cattle		
	5	−	−	−	+	+	+	+	−	+	+	+	−	+	−	Cattle		
	6	−	−	−	+	+	+	+	+	−	+	+	−	+	−	Cattle		
	7	−	− or +	−	+	+	+	+	+	+	+	+	−	+	−	Cattle		
	8	+	−	−	+	+	+	+	−	+	+	+	−	+	−	Cattle		
	9	− or +	+	−	+	+	+	+	−	+	+	+	−	+	−	Cattle		
Br suis	1	−	++	+	+	+	−	−	+	−	−	−	+	+	+	Pigs	Red	Red
	2	−	−	−	+	+	−	−	+	−	−	+	+	+	−	Pigs hares		
	3	−	−	+	+	+	+	+	+	−	−	+	+	+	+	Pigs		
Br ovis (n sp)		+	−	+	+	+	±	+	−	−§	−	−		−		Sheep	Blue	Red

Species differentiation is obtained on albimi or tryptose agar with the following graded concentrations of dyes from National Aniline Division Allied Chemical and Dye Co New York 1 75 000 (a) 1 50 000 (b) 1 100 000 (c) thionin and 1 50 000 (b) 1 100 000 (c) basic fuchsin *Other concentrations may be preferable with other growth media* Interpretation of results should be controlled with the reference strains of each species (*Br melitensis* 16M *Br abortus* 544 *Br suis* 1330)

† Four substrates that provide for differentiation of the three species are given in the table It is recommended however that all substrates be used for examining atypical strains

‡ Usually positive negative varieties occur

§ r" (rough) antigen present in all *Brucella* strains

[1] Modified from Report of Special Committee on Taxonomy of Brucella

from animal tissues or exudates, whereas the bacillary form of *suis* and *abortus* prevail under the same conditions The organisms are variable in length 0 3 to 2 3 microns

The detection of brucellae in heavily infected material such as placental specimens and tissues at necropsy can be carried out by the modified Køester stain* and the modified Ziehl Neelsen method † The brucellae, often located inside of cells retain the primary stain

Morphology of the organisms in their intracellular location in phagocytic cells may be observed in cell culture preparations by the above staining methods after thinly overlayering the preparation on the slide with agar which preserves the morphology of the host cell

The brucellae are aerobic and grow more abundantly when liquid media are thoroughly aerated by shaking or sparging (Glassman and Elberg, 1946) Heavy suspensions of cells can be obtained by laboratory devices such as vigorous agitation or growth in a cellophane sac containing medium which in turn is immersed in simple isotonic fluid containing an adsorbent such as charcoal (Sterne, 1958) Such devices allow high yields even from small inocula *However additional aeration is not required* for primary isolation from infected material In fact when the inoculum contains only a few organisms initiation of growth is achieved most satisfactorily under conditions of reduced air supply (Gerhardt 1958)

Br abortus usually requires for primary isolation 5 per cent carbon dioxide in the gaseous environment whereas *Br suis* and *Br melitensis* will grow without this supplement (Wilson 1931) Although the major products of CO fixation by *abortus strains* are pyrimidines glycine and alanine there is no direct explanation of the CO_2 requirement other than the suggestion that pyrimidine biosynthesis from CO may be more vital in those brucella strains requiring additional CO_2 than is the case for example of *E coli* which also fixes CO_2 but does not require added CO (Newton Marr and Wilson 1954)

Culture media containing commercial peptones such as trypticase soy' and albimi support growth of the various species and strains Growth can be obtained in meat infusion or potato infusion media supplemented by 10 per cent serum Synthetic media have been devised but will not support the growth of all strains Nutritional requirements have been reviewed by Gerhardt (1958) and Wundt (1961)

Primary isolation from animal or human tissues is sometimes complicated by the contamination of the specimen with other types of microbes The crystal violet medium of Kuzdas and Morse (1953) has been of great value in the isolation of brucellae from such contaminated specimens This medium is inhibitory for some strains but this may be overcome by omitting the crystal violet The technic of Castaneda (1947) employing the biphasic broth agar system, is almost ideal for culturing small numbers of brucellae from blood specimens Antibrucellar toxicity of peptones caused by elemental sulfur arising from the degradation of sulfur-containing amino acids (Schuhardt Rode Oglesby and Lankford 1950) has caused much difficulty in the past The addition of charcoal or the use of semisolid broths seems to help in neutralizing this toxicity Newer methods of peptone production have all but eliminated the problem

As mentioned above the oxidative activity of strains on various substrates is now widely used to supplement fermentation tests *in classifying brucella strains* The pattern of oxidation of amino acid and carbohydrate substrates seems to be sufficiently typical of species and their biotypes to characterize the strain irrespective of discrepant serologic and biochemical reactions (Meyer 1961 Meyer and Cameron 1961) *Br suis* differs qualitatively from the other two species in that it also oxidizes the 4 amino acids of the urea cycle *Br suis* also consistently displays the highest rates of utilization of the carbohydrate substrates The production of H S from sulfur-containing amino

*After heat fixation stain for 1 minute with newly prepared mixture of 2 parts saturated safranin and 5 parts normal KOH solution wash decolorize with 0 1 per cent of H_2SO_4 for 10 to 20 secs wash and counterstain with methylene blue solution

†After heat fixation stain 10 minutes with 1 10 Ziehl Neelsen carbol fuchsin wash treat with 0 5 per cent acetic acid for 30 secs wash and stain with methylene blue

acids in peptones is also a useful taxonomic quality *Br suis* (type I) varieties are active in this respect continuing to produce H S on solid media for at least 4 days the 3 types of *Br abortus* are next in H_2S productivity Occasionally a strain of *Br melitensis* will produce some H S but for at most 1 day (This determination is usually made by daily placement of lead acetate impregnated filter paper in the air space over the agar slant) The pH range of urease activity expressed in terms of pH threshold (range pH 2 to 7) has been studied by Van Drimmelen (1961) with apparently useful results on South African strains

MUTATION

Brucellae undergo population changes during growth on laboratory media producing colonies of the smooth the intermediate the mucoid and the rough varieties (Marshall and Jared 1931) These colonial types as viewed by oblique reflected light (Henry 1933) appear as typically smooth transparent entire blue green tinted colonies (S) an intermediate (I) and/or mucoid (M) form of diminished virulence (Jones and Berman 1951) and the rough type (R) of minimal virulence The progressive changes S→R show increasing degrees of opacity and tinctorial trends to a brownish yellow The smoothness of the colonial phases can be tested on glucose glycerol or serum dextrose agar and by the application of the dyes crystal violet and 2 3 5 triphenyltetrazolium to the colonies (White and Wilson 1951 Huddleson Richardson Warner and Baltzer 1952) The tinctorial character of the colony is a direct function of the antigenic composition of the organisms and the reaction of the surface antigens with the dyes Antigenically nonsmooth cells are detected by their ready agglutinability in a drop of 1/1 000 acriflavine solution performed as a slide agglutination test True smooth colonies (S) remain as a uniformly turbid suspension in the drop intermediate types (I) give varying results rough (R) colonies flocculate and mucoid (M) colonies show a slimy and threadlike flocculation So-called smooth R types (S^R) and smooth M types (S^M) give the reaction characteristic of R and M colonies respectively

Metabolites accumulating in the medium have long been thought to influence the colonial variation of the organisms The selective ability attributed to accumulation of D alanine as expressed in the culture by predominance of nonsmooth variants is suppressed by hydrogen acceptors such as nitrate ion methylene blue and resazurin reducing the iron content of the medium to trace amounts causes the predominance of highly virulent smooth types This is probably due to suppressed synthesis of heme enzymes (Altenbern *et al* 1957 Waring *et al* 1953 Reusse 1961) Conversely incubation of smooth cultures under reduced atmospheric tension gives predominance of rough types (Braun *et al* 1956) The metabolic pathway leading to the predominance of one or another colonial and antigenic type is not susceptible to single factor analysis and is clearly far more complex than the experimental designs would indicate

The complexity of the causes of smooth→nonsmooth colonial changes is further reflected in similarly directing actions of pH Eh temperature and penicillin besides those mentioned above Conversely degradation products of deoxyribonucleic acid favor selection of smooth virulent types (Braun 1956)

Certain mutants compete successfully with the parent strain in the tissues of the guinea pig The ability of colonial variants to establish themselves in the host in competition with the smooth type depends on the duration of the S type infection in the later periods of S-type infection the hypersensitivity reactions operate less efficiently against nonsmooth mutants thereby increasing the ability of the latter to survive in competition with the smooth mutants The fact that less virulent nonsmooth types are able to persist in localized abscesses may be a result of the protection afforded such organisms against the tissue sterilizing mechanisms or of the local accumulation of metabolites or of anoxia favoring the selection of these mutants Accumulation of necrotic material containing deoxyribonucleic acid degradation products would tend to maintain some population of virulent cells also The phenomenon of in vivo selection of mutants is most complex

from animal tissues or exudates, whereas the bacillary form of *suis* and *abortus* prevail under the same conditions The organisms are variable in length 0 3 to 2 3 microns

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cysteine hydrochloride (ZøBell and ZøBell 1932) thus permitting dilutions for making viable cell counts

Destruction of brucellae by pasteurization occurs easily even if the milk is heavily contaminated They are also quickly destroyed by hypochlorites phenol formaldehyde and the quaternary nitrogen compounds area disinfection is accomplished readily with quaternary compounds such as Roccal or Zephiran

No loss of viability immunogenicity or colonial variation occurs during storage for at least 225 days at 0 C after lyophilization (Heckly *et al* 1961 Hutton *et al* 1951 Van Drimmelen and Steyn 1958) Aging of the cells before the freeze drying process improves their survival This has been shown to be due to the accumulation in the culture fluid of a waxlike substance of a lecithin nature (Bergman *et al* 1957) Exposure of *Br abortus* to 2 curies per liter of broth medium fails to sterilize the culture although stimulation of polymorphism and temporary inhibition of growth occurs (Ryagrizov and Litvinov 1960) Exposure of suspensions of *Br abortus* containing from 10^8 to 10^{10} cells per ml to x irradiation ranging from 750 to 1 500 kiloroentgens completely inactivates the suspensions (Beutel 1962)

ANTIGENIC COMPOSITION

Antisera prepared against the smooth form of any one of the 3 species agglutinate homologous and heterologous strains equally Sera absorbed with a heterologous antigenic suspension act monospecifically on the homologous antigen albeit with some loss of activity (Evans 1923) Such monospecific sera serve as important diagnostic reagents However it is not possible with such methods to prepare a monospecific *Br suis* antiserum because the serologic relation of *Br suis* and *Br abortus* is too close Some indication that antigenic individuality exists in *Br suis* has come from gel diffusion patterns in which a particular band of diffusing antigen appears to be limited to strains of *Br suis* Antisera can be exhausted of agglutinins by absorbtion with large doses of heterologous suspensions smaller doses leave the agglutinins specific for the homologous suspension In studying the kinetics of the agglutination test Miles (1933) showed the existence of two optimal ratios indicating the existence of at least two antigens on the surface of the cells

A monospecific antimelitensis serum agglutinates only *Br melitensis* due to its M antigen content yet fails to agglutinate *Br abortus* which also possesses some M antigen The M antigen on *Br abortus* is apparently insufficient in amount or distribution for agglutination to occur The agglutination of *Br abortus* by an unabsorbed antimelitensis serum is presumably due to the abortus system of antigens Miles (1933) postulated that the brucellae contain the two antigens A and M in different proportions characteristic of the two major antigenic species *abortus* and *melitensis* the abortus surface would be characterized by an A M antigen ratio of 20 1 Quantitative precipitin reactions support these claims (Pennell and Huddleson 1938 Silverman and Elberg 1950) but also suggest the existence of additional antigens as was indicated by the behavior of the native antigen of *Br suis* prepared by Miles and Pirie (1939)

Gel diffusion studies on soluble antigens distinguish the main antigenic varieties (Redfearn and Berman 1960) As many as 36 precipitinogens have been detected in preparations from *Br melitensis* (Glenchur *et al* 1962) Such bands are distributed among strains in quantitatively different ways so that a particular antigen might be easily detected in virulent strains but not detected in attenuated strains but the method thus far has contributed little information of taxonomic value Cell wall preparations of *Br suis* remove agglutinating activity in parallel with the removal of mouse protective activity from rabbit sera Protective ability of a serum was measured by its ability to eliminate the brucellae from the spleens of mice within a week or two after infection

The cell walls are more effective immunizing agents for mice and guinea pigs than the corresponding whole cells as measured by the numbers of brucellae in the

because of the large number of variables involved (Braun *et al* 1951)

Variations in brucellae may also be due to the action of bacteriophage Pickett and Nelson (1951) first described the isolation of phages active against brucella during their studies on the production of L forms in blood cultures and stressed the importance of these forms in pathogenesis Using phages isolated in the U S S R Ostrowskaya and others observed that the antigenic activity of phage resistant attenuated variants of *Br abortus* strain 146 is weaker than that of the parent strains The variant strains contain more polysaccharide and less DNA and proteins proportionally than do parent strains the polysaccharides are composed of more uronic acid and sugars than in the parent strain and the mutants acquire arabinose and xylose in their make up (Dubrovskaya Ostrovskaya and Glubokina 1958 Dubrovskaya and Ostrovskaya, 1960) Cells carrying phage are extremely mucoid in colonial appearance (Jones McDuff and Wilson 1962) are reduced in their urease activity and H S production and are much less virulent for guinea pigs but retain their original dye sensitivities It is not yet clear whether true lysogeny occurs The strange geographic distribution of phages and of lysogenic strains of brucellae may parallel the geographic distribution of the species themselves (Jones, McDuff and Wilson, 1962)

Species transformations in vitro have been looked for in culture collections for many years Huddleson (1961) studied the possibility of changing in the laboratory the biochemical and the antigenic characteristics used in speciation but could find no evidence for it however cells of the various species could develop resistance to dyes formerly inhibitory to them Earlier experiments on parabiosis by Lisbonne *et al* (1938) later developed by Lederberg in transduction experiments purported to show compatibilities and incompatibilities between the growth of two species of brucellae separated only by parlodion membranes Lisbonne claimed also to have demonstrated the induction of H S production in *Br melitensis* growing parabiotically with *Br suis* initially the only sulfide producer of the pair These results bear re examination in the light of the advances in methods and concepts of the last decade

STERILIZATION

Brucellae are killed by direct sunlight but are easily protected from ultraviolet wave lengths by the common vehicles in which they are discharged from the host In pastures and barnyards they have survived from 65 days to 182 or more days in dead fetuses and fetal membranes (Bosworth 1934 35)

In dry soil and protected from sunlight *Br melitensis* has survived up to 69 days *Br abortus* survive freezing temperature over 824 days in bovine urine lake water tap water raw milk bovine feces and soil At temperatures of 18 to 22° C an initial population of 500 million cells per ml of *Br melitensis* in unsterile water fell in 60 days to 10 000 per ml (Ogarkov 1962)

In cheeses made from contaminated milk of goats and sheep by rennet coagulation *Br melitensis* survive more than 2 months but less than 3 months as demonstrated by inoculation into guinea pigs (Gargani 1952) Goat and sheep cheeses are most serious sources of infection when unpasteurized because they may contain enormous numbers of virulent *melitensis* organisms in countries of the Middle East they are responsible for epidemics of human brucellosis in the spring when the flocks are brought down from the hills and the accumulated stores of cheeses are distributed in the towns and villages Brucellae from contaminated milk are killed within a few days during the aging process in cheese but survive up to 11 days in butter

Under experimental conditions 45 per cent of the organisms survive from the aerosol state for 24 hours at 20 C and 12 per cent relative humidity The brucellae will survive 6 months or longer with little loss if the cell suspensions are added to ascorbic acid gelatin mixtures or dextrin solution whereas they begin to die off 10 minutes after being suspended in phosphate solutions and in physiologic fluids such as Ringer Locke and Tyrode In contrast they survive in a mixture of inorganic salts and

in the uterus the genitalia skeletal structures and in the reticuloendothelial system with the occurrence of abscesses for example in the spleen The Danish strain of *Br suis* does not appear to be a human pathogen

In cattle infection by *Br abortus* produces widespread necrosis and exudation in the placenta and uterine carunculae Death of the fetus results possibly from interference with fetal circulation by the inflammatory process and abortion occurs after necrosis and separation of cotyledons Localization in the supramammary lymph nodes leads to excretion of organisms in the milk

The disease of sheep produced by *Br ovis* is characterized by lesions of the epididymes the tunicae vaginales and the testes in the ram and by placentitis in the ewe with abortion or neonatal death of lambs

Brucellosis in the reindeer of northern Siberia and the transpolar region appears to have been introduced by cattle Abortions fever depression and death may follow infection of reindeer by all of the brucella species Joint and ligamental lesions bursitis mastitis and metritis orchitis and epididymitis were also reported by Cherchenko (1961) However bacteremia is not frequent Apparently only animals over 6 to 12 months of age are susceptible to the clinical disease although young animals are probably infected within the first 3 to 4 months of life The disease is most prevalent in the autumn and the spring The strain responsible for this animal disease seems not to be highly pathogenic for man

Experimental infections are easily induced by many different routes in the guinea pig the rabbit the mouse the monkey the cotton rat and the hamster The guinea pig is the most susceptible in terms of minimal numbers of bacilli required for infection by the subcutaneous route Differentiation of species by the lesions they produce in the various animal species has been claimed but the great variation in virulence among strains and the variation in susceptibility among individual animals makes this a questionable procedure

Granted that the picture in the guinea pig varies with the strain the species and the dose injected one usually observes—4 to 5 weeks after subcutaneous injection—swollen lymph nodes draining the injection site enlarged and engorged spleen and some scattered grayish foci in the liver Testicular lesions may be observed The spleen and/or the lymph nodes often are the only sites from which the organisms can be recovered in the guinea pig *Br melitensis* infections are most virulent for guinea pigs often leading to death

The hare has received much attention during the past several years because it acts as a reservoir of infection in nature (Bouvier *et al* 1954) Bendtsen *et al* (1956) presented evidence that infection of swine in Denmark is maintained by hares which carry *Br suis* in necrotizing lesions of the testicles the uterus the mammary glands the spleen the lungs and the liver as the principal organs of localization Taran *et al* (1962) failed to isolate brucellae from 200 gerbils (*Meriones meridianus*) collected under natural conditions confirming thereby the improbability that this rodent constitutes a natural reservoir

The role of ticks in the spread of brucellosis is widely discussed Rementsova (1962) has summarized the world wide studies on brucellosis of wild animals and has claimed that arthropods and insects transfer the infection to wild rodents which act as permanent reservoirs However studies on ticks fleas flies etc for carriage and transmitting ability have yielded conflicting results The view that farm animals are in fact the main spreaders of their own infection still retains the greatest plausibility (Taran 1960) The role of ticks seems improbable in the light of their ability to eliminate brucellae from their tissues despite their infectiousness during metamorphosis Their role if any is probably mechanical rather than biologic (Gudoshnik 1955)

PATHOGENESIS

Although the infectious process undoubtedly involves complex pathways it is possible to derive a few general principles from the picture of brucella infection in the various host species *Br abortus* infection in the pregnant bovine is perhaps most thoroughly studied and serves as a model for the genus The description is based on the elegant work of Payne (1959)

Following a conjunctival infection (not

spleens of immunized animals 7 days after challenge (Foster and Ribi 1962, Markenson Sulitzeanu and Olitzki 1962) Furthermore the cell walls of organisms obtained from infected animals seem to contain materials which contribute to intracellular survival and growth these materials may be important immunogens but have not yet been produced in vitro (Smith and Fitzgeorge, 1964)

Glenchur *et al* (1963) found that the soluble constituents of brucella induce antibodies active in agglutination precipitation blocking reactions and cutaneous hypersensitivity The antigens obtained by phenol extraction (Miles and Pirie 1939 Plescia *et al* 1961) or by trichloracetic acid precipitation (Dubrovskaya 1951 1954) contain phospholipids, carbohydrate a formyl derivative of an amino polyhydroxy compound which may be an amino sugar and a DNA-rich fraction The polysaccharides obtained by Dubrovskaya (1951) are composed of glucose galactose glucosamine and hexuronic acids there is at present no evidence that these polysaccharides account for species specificity

In addition to the major antigenic constituents of the smooth forms mucoid and rough variants possess antigens dominant for the respective phases these antigens seem to be deeply situated in smooth cells However these do not interfere with the A and M antigens when the latter are dominant as in the smooth phase

Pasteurella, Salmonella, Vibrio and *Brucella Spp* seem to possess certain antigenic determinant groups in common a fact which may account for a certain amount of cross reaction when sera from fevers of undetermined origin are being studied for etiologic identification Proper use of the agglutinin absorption test will readily identify these co reacting antibodies

INFECTION SPECTRUM

Brucellosis is primarily a zoonosis a disease of animals transmissible to man but not transmissible from man to man in nature The most pathogenic species for man are *Br melitensis* and *Br suis* although many strains of *Br abortus* also have high human virulence Each species contains strains expressing the full range of virulence avirulence for both animals and man In view of the fact that the transmission of brucellae from one animal species to another and from one geographical area to another is in an active stage of evolution, it is unwise to describe the spectra of infection for each brucella species too narrowly and it is important to realize that the catalogue of natural hosts is still evolving

Br melitensis infection is the most important cause of brucella infection throughout the world in goats sheep and reindeer This species has also been isolated from camels (Africa) cattle swine wild hares fowl and wild guinea pigs

Br abortus is the principal cause of infection in cattle camels (U S S R) gazelles (Egypt) water buffaloes and horses It has also been isolated from dogs swine wild hares wild rats chamois wild deer elk moose ground squirrels field mice and bison

Br suis is the principal cause of brucella infection of swine and wild hares It has also been isolated from horses wild rats cattle jack rabbits and dogs

In the goat infected with *Br melitensis* the febrile period begins 3 to 4 days after infection and the bacteremic phase occurs within the first week There are very few signs of continuing infection regular enough to serve diagnostically Abortion following a primary attack may occur throughout the flock in subsequent years abortions are observed infrequently Such signs as roughness of the coat lameness and mastitis may occur but the infection more frequently may be clinically silent as it progresses through the flock yet the infection remains active and organisms are shed in milk urine feces and vaginal secretions Principal sites of brucella localization in the infected animal are the lymph nodes the mammary glands the pregnant uterus and the kidneys Man becomes infected via skin contact with the infected tissues or discharges from the animal consumption of raw milk and oth r unpasteurized dairy products inhalation of contaminated dust and contact of the mucous membrane with infected materials such as manure feed and residues (Renoux 1957)

Br suis infections are frequently localiz d

in the uterus the genitalia skeletal structures and in the reticuloendothelial system with the occurrence of abscesses for example in the spleen The Danish strain of *Br suis* does not appear to be a human pathogen

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Experimental infections are easily induced by many different routes in the guinea pig the rabbit the mouse the monkey the cotton rat and the hamster The guinea pig is the most susceptible in terms of minimal numbers of bacilli required for infection by the subcutaneous route Differentiation of species by the lesions they produce in the various animal species has been claimed but the great variation in virulence among strains and the variation in susceptibility among individual animals makes this a questionable procedure

Granted that the picture in the guinea pig varies with the strain the species and the dose injected one usually observes—4 to 5 weeks after subcutaneous injection—swollen lymph nodes draining the injection site enlarged and engorged spleen and some scattered grayish foci in the liver Testicular lesions may be observed The spleen and/or the lymph nodes often are the only sites from which the organisms can be recovered in the guinea pig *Br melitensis* infections are most virulent for guinea pigs often leading to death

The hare has received much attention during the past several years because it acts as a reservoir of infection in nature (Bouvier *et al* 1954) Bendtsen *et al* (1956) presented evidence that infection of swine in Denmark is maintained by hares which carry *Br suis* in necrotizing lesions of the testicles the uterus the mammary glands the spleen the lungs and the liver as the principal organs of localization Taran *et al* (1962) failed to isolate brucellae from 200 gerbils (*Meriones meridianus*) collected under natural conditions confirming thereby the improbability that this rodent constitutes a natural reservoir

The role of ticks in the spread of brucellosis is widely discussed Rementsova (1962) has summarized the world wide studies on brucellosis of wild animals and has claimed that arthropods and insects transfer the infection to wild rodents which act as permanent reservoirs However studies on ticks fleas flies etc for carriage and transmitting ability have yielded conflicting results The view that farm animals are in fact the main spreaders of their own infection still retains the greatest plausibility (Taran 1960) The role of ticks seems improbable in the light of their ability to eliminate brucellae from their tissues despite their infectiousness during metamorphosis Their role if any is probably mechanical rather than biologic (Gudoshnik 1955)

PATHOGENESIS

Although the infectious process undoubtedly involves complex pathways it is possible to derive a few general principles from the picture of brucella infection in the various host species *Br abortus* infection in the pregnant bovine is perhaps most thoroughly studied and serves as a model for the genus The description is based on the elegant work of Payne (1959)

Following a conjunctival infection (not

unlikely in nature) bacterial proliferation occurs within 1 week in the regional lymph glands Passage of organisms through the lymphatic system to the bloodstream is followed by splenic infection and spread to other organs such as supramammary nodes and uterus Within a month of infection *Br abortus* localizes in the interstitial connective tissue in the uterine wall then from the uterine lymphatics progresses to the lumen of the uterus with striking bacillary multiplication in the pregnant uterus From the uterine lumen the organisms gain access to the area between the placental masses of blood vessel plexuses (the cotyledons) and the allantochorionic membrane the bacilli multiplying enormously in the chorionic trophoblast cells and in the placental tissue This intracellular proliferation of brucellae probably leads to production of bacterial endotoxins which in turn may cause the abortion (Urbaschek 1961) The actual selective multiplication in fetal tissue has been attributed to the presence of erythritol a polyalcohol which stimulates growth of brucellae (Williams *et al* 1962) This hypothesis is strongly supported by isolation of erythritol from tissues in amounts correlating with their susceptibility and by its ability to enhance systemic calfhood infections by brucellae

The chorio-allantoic connective tissues and the fetal blood vessels are invaded next then the placental fluids and the fetal spleen Fetal stomach and lungs are infected at or near the time of abortion

This picture of initial lymphatic infection spreading to lymphoid tissue with further extension via the blood constitutes the central theme of pathogenesis with several variations observed in the individual host species

The pregnant but not the nonpregnant uterus is selectively invaded in both bovine and rabbit (Payne 1959) However progesterone does not seem to be a factor in the increased susceptibility of the uterus (Payne 1960) although it does enhance systemic invasion and multiplication in the pregnant bovine and rabbit (Kniazeff and Elberg 1964)

The tissue response is proliferative The basic lesion is a granuloma composed of epithelioid cells and lymphocytes it is independent of the existence of hypersensitivity for its formation Giant cells of the Langhans and the foreign body variety may also be included in the lesions of the lymphatics the spleen the liver bone marrow and other areas of localization The histopathologic response to brucellar infection with the exception of the reaction at the injection site remains qualitatively the same despite the size of the inoculum and heals in about the same time again independently of the infecting dose However the extent and the severity of the response is a function of dose (For description of the genesis of these reactions see von Albertini and Lieberherr 1937 Braude 1951 Elberg *et al* 1955 McCamish and Elberg 1962)

Brucellae are intracytoplasmic during a large period of the infection localizing in the mesenchymal and the ectodermal tissues (Meyer 1943) They are abundant within macrophages fibroblasts endothelial and reticular cells interstitial cells of the testes and alveolar cells

When cell cultures of histiocytes of the inflamed peritoneum or from the pulmonary tree are infected virulent strains multiply intracellularly in normal histiocytes but not in immune histiocytes (Pomales Lebron and Stinebring 1957 Elberg *et al* 1957) When the rabbit histiocyte (peritoneal or alveolar) is employed under conditions which promote growth and multiplication of the cells immune serum is necessary in the culture fluid for resistance to the cytotoxic action of the brucellae and for suppression of bacterial growth Under other culture conditions and using other peritoneal inflammatory stimulants which affect the function of the histiocytes (Elberg *et al* 1960) the serum appears not to be required (Holland and Pickett 1958)

The intracellular events which take place during inhibition of brucella growth by histiocytes have been explored only recently (reviewed by Elberg 1960) Histiocytes contain a lysozymelike substance which acts on the cell wall Living brucellae are extremely resistant to this material but are rendered susceptible to it when first treated with glycine Histiocyte extract glycine brucella systems cause lysis and death of the brucellae (Ralston and Elberg 1961)

Glycine action in histiocytes may be enhanced by the addition of specific immune serum The susceptibility of strains to this intracellular killing is correlated inversely with their virulence These studies do not prove that histiocytic lysozyme is involved in the destruction of brucellae in infectious processes however the fact that a simple amino acid such as glycine can render the cells susceptible suggests that other metabolites may alter wall structure and consequently resistance to tissue enzyme(s) (Stinebring 1962) Ultimately the demonstration of a role for lysozyme if any does exist will depend on the reconstruction in vitro of a multiplicity of factors each of which might be important in potentiating stepwise degradations of the bacterial surface (Ralston Baer and Elberg 1961)

The brucella endotoxin operates in the causation of illness in a manner similar to that of other gram negative endotoxins (Spink 1956) Clinical complications such as vascular collapse and secondary shock following treatment with antibacterial drugs would appear to result from endotoxin liberation

Spink (1957) regards the symptomatology of brucellosis as conditioned in large measure by the development of hypersensitivity to the bacterial products and by allergic inflammatory reactions locally and systemically In his view the incubation period reflects the time required for hypersensitivity to develop When this hypersensitivity is present from a prior exposure reinfection leads to an incubation period measured in hours rather than days due probably to the accelerated lysis of the organisms intracellularly and the liberation of endotoxins which trigger further allergic and toxic reactions Absence of hypersensitivity may provoke prolonged intracellular parasitism in the reticuloendothelial system without much disturbance of these cells or too rapid a release of endotoxins thus encouraging a more or less symptomless infection

CLINICAL PICTURE

The disease ranges from a completely symptomless state to a fulminating fatal malady More than 150 different symptoms are on record making differential diagnosis extremely difficult at times The incubation period ranges from a few days to a few months averaging between 8 and 30 days the variation apparently depending on the size of the infecting dose and the previous history of sensitization to the organism

The onset of the disease may be either abrupt with chills and fever or insidious Chills weakness fatigue exhaustion severe frontal headache and backache are frequently encountered At night profuse sweats and generalized aching of joints occur especially in the back of the neck and the lumbosacral area Fever of 101° to 105° F may be reached during the first week rising in the late afternoon and the early morning hours and concluded by drenching perspiration of characteristic odor Bronchitis may occur following direct inhalation infection (Meyer and Eddie 1949)

Frequently abdominal pain is present probably due to mesenteric lymphadenitis or accompanying enlargement of liver and spleen According to Janbon and Bertrand (1956) alterations in the liver are a regular feature of septicemic brucellosis Aspiration biopsy of an enlarged and tender liver may reveal a simple hepatitis Capillary congestion Kupffer cell reaction and beginning granulomas characterize the microscopic picture Spontaneous recovery is usual but failing this the changes evolve toward hemorrhagic subacute splenohepatitis with well developed granulomas In the malignant form hepatic necrosis may occur Chronic infection of bile ducts commonly occurs resulting in pain or jaundice

As the illness progresses anorexia nausea vomiting and mental depression may be present Following hematogenous spread secondary areas of localization may lead to endocarditis (Peery and Belten 1960) renal necrosis and pyelonephritis (Greene *et al* 1952) various forms of nervous system brucellosis such as meningoencephalitis subarachnoid hemorrhage myelitis myelopathy and neuritis (cranial and peripheral) as well as psychiatric changes (Fincham *et al* 1963) Skeletal localizations such as spondylitis and osteomyelitis (Kelly *et al* 1960) support the idea that no organ system is immune from infection Personality

changes and anxiety states occur commonly in protracted disease Abortion may occur in the human, although localization of the organism in the uterus is a much rarer event in pregnancy of the human than in other susceptible host species (For further discussion of the clinical aspects of brucellosis see Spink 1956 Dalrymple Champneys 1958)

The infection in humans usually subsides gradually and recovery sets in There are often residual manifestations of an allergic like nature and other irreversible changes such as bone and joint lesions and impaired liver function (Naumov 1962)

Chronic brucella infection presenting as fever of undetermined origin occurs frequently following *Br suis* infection and it may be difficult to confirm the diagnosis even when careful bacteriologic serologic or pathologic examinations are made Recurrent chills and fever may extend over many years with localization of organisms in calcified necrotic lesions

In summary Persons with a bacteremic type of infection are usually acutely and severely ill Those who develop only rising serologic titers and from whom no organisms can be isolated show acute or subacute systemic manifestations (Bothwell 1962) In the experience of Martin *et al* (1961) this group numbers three times as many patients as are to be found in the bacteremic group Those who develop a localized type of disease yield positive cultures only when specific tissue is cultured Their sera may be negative or give only low titers Finally there may be latent completely symptomless infection

LABORATORY DIAGNOSIS

The only definitive diagnosis for brucellosis is the isolation of the causative organism This is accomplished most frequently from the blood although cultures may also be obtained from sputum biopsy or aspirated material from bone marrow lymph nodes or other tissues urine and occasionally bile cerebrospinal fluid and stools Since the organisms may only occur sporadically in the blood many successive specimens may have to be studied before culture is successful Blood cultures should be made in pairs with one set incubated at 37° C in the presence of 5 to 10 per cent additional CO for several weeks Subculture should be made frequently onto solid medium to isolate the typical smooth circular translucent, smooth-edged colonies which appear blue green and granular of surface when observed by oblique reflected light

Fluorescent labeled brucella antisera and labeled anti human gamma globulin have helped the direct visualization of the organism in human clinical specimens (Biegeleisen *et al*, 1962a) When a specimen is examined after treatment with labeled anti brucella serum or with unlabeled antibrucella serum followed by labeled anti human gamma globulin, the fluorescent bacterial cell walls can be seen under ultraviolet illumination This procedure is especially useful in examination of impression smears of tissues (Biegeleisen *et al* 1962b)

The tube agglutination test is the best standardized serologic procedure Dilutions of test serum from 1 10 to 1 5 120 reveal the agglutinating antibody in the overwhelming number of cases the tests should be run against control positive sera calibrated against standard sera and cultures obtainable from brucella reference laboratories designated by the World Health Organization Only organisms proved to be in the smooth phase should be employed as agglutinatin antigens Sera for tests should be taken *before* not after a skin test has been given in order to avoid the complication of possible antibody formation by an antigenic skin test reagent

Tests should be made for the presence of incomplete or blocking type antibodies in cases with a strong presumptive clinical diagnosis of brucellosis despite a negative tube agglutination This can be done by (1) performing the test in a diluent of 5 per cent sodium chloride instead of 0 85 per cent (2) using a protein diluent such as plasma albumin in place of saline (3) adding to all tubes in the series an equal volume of a dilution of positive serum which is known to agglutinate at that dilution (4) developing a positive test by adding the Coombs reagent (anti human globulin serum) to

the nonagglutinating preparations and (5) centrifuging the tubes before making the reading

Since a positive serum is a matter of titer determination attention must be paid to the level of agglutinins in the population of the area in general Usually titers of 1 160 or higher are indicative of infection and more respect is accorded to sera the titers of which are in the higher ranges (Castaneda 1961)

The agglutination pattern in a group of 60 cases of acute brucellosis studied by Cluff *et al* (1957) revealed three distinct patterns of antibody response One showed an abrupt rise in titer with the acute infection followed by a fall to below 1 100 The second showed a rising titer with the development of acute disease and a maintenance of the titer (over 1 100) during subsequent years The third showed multiple elevations with intervening periods of depressed titer the secondary elevations often accompanied by symptomatic relapse secondary localizations and abscess formation

Modifications of the agglutination test have been developed for the detection of animal infections Sensitive antigens plate tests and incubation at various temperatures all have been incoporated in various systems to make the tests more specific and to adapt them for mass screening surveys (Stableforth 1953) Cross reactions can be resolved and the primary agent identified by appropriate cross absorptions of the sera

The prozone phenomenon wherein no agglutination occurs in the higher concentrations of serum antibody has been ascribed in part to the presence of blocking type antibody which is often present in chronic infections (Zinneman *et al* 1959) Agglutinating antibodies are distributed between the gamma and the beta globulins blocking type antibodies occur first in the gamma fraction and then in the beta globulins later in the infection They belong to the 4 5 to 5 6 S whereas the agglutinins are 6 5 S globulins (Aznar Reig and Lopez, 1960 Zinneman *et al* 1961) In serum of immune pigs agglutinins against *Br suis* are in the gamma and the beta globulin fractions These agglutinins belong to both 7 S and 18 S types the 7 S type being stable at pH 2 5 to 3 at which 18 S type brucella antibody is destroyed (Franěk *et al* 1962) *Br abortus* agglutinins of hyperimmune rabbits are also in the light component none being detectable in the macroglobulin (Hemmings and Jones 1962)

Sera of patients with chronic type abscesses also show precipitins which are usually absent in acute case sera (Glenchur and Seal 1962) Patients infected for less than 6 months usually have agglutinating type antibody rather than complement fixing type antibodies in their serum a relation which seems to reverse itself after 6 months of infection (Foz and Garriga 1954) Blocking type antibodies can participate in the complement fixation reaction which may help to explain the higher fixation activity of sera later in the course of the infection

The complement fixation test has been most useful in bovine and sheep brucellosis Blagobetschtschenskaya (1954) and Yuskovets (1954) reported that they could distinguish the serologic reactions of vaccinated animals from those of naturally infected animals Hill (1963) has confirmed this finding thereby allowing much greater freedom in the study of prevention and control measures previously regarded as unacceptable if they led to persistently positive agglutination reactions For the diagnosis of animal brucellosis modifications of the agglutination test which reveal the presence of agglutinins in milk samples have been used widely and include the milk ring test, the plate agglutination test and whey agglutination tests (McDiarmid 1958) (A review of diagnostic tests for bovine brucellosis has been presented by Hill 1963)

An intracutaneous test with brucella preparations provides information on the allergic state in 24 to 72 hours A positive reaction in brucellosis like a positive tuberculin reaction indicates that the host has had at some time an experience with the antigenic components of the brucellae However it does not indicate the current status of infection Several preparations are available for the study of cutaneous hypersensitivity They are composed either of heat killed brucellae of culture filtrates or of

partially purified cell extracts When adequately purified they are not antigenic are active in very high dilution and reveal the hypersensitive state very early after infection

A comparative study based on response to dosage was carried out in the guinea pig with several preparations to determine the sensitivity and the antigenicity of the reagents The reagents prepared by Benedict (1953a b) Fust *et al* (1949) and Ottosen and Plum (1949) were devoid of antigenicity and highly reactive indicators of sensitivity (The subject is reviewed by Burki 1961)

Studies on the correlation between skin test sensitivity and other tests for sensitivity employing cells and tissues maintained under *in vitro* culture conditions suggest that the latter tests may be much more sensitive as indicators of hypersensitivity (Stinebring *et al* 1958)

TREATMENT

The current status of therapy has been summarized by Spink (1960) Supportive measures such as minimal physical activity bed rest symptomatic relief of pains and headaches and sedatives during the acute phase are utilized Antibiotics suppress the infection and thereby lowers the chances of secondary localizations and the development of excessive hypersensitivity The tetracycline drugs are preferred the optimal regimen being 500 mg orally every 6 hours for 21 days This schedule may be repeated if relapse occurs in 6 to 8 weeks In severe infections 1 to 2 Gm of streptomycin daily for 14 days is administered intramuscularly in addition to the tetracycline therapy Peripheral vascular collapse may occur following the first course of antibiotics apparently due to liberation of endotoxin in an already hypersensitive person. The use of corticotropin or glucocorticoids with the antibiotic prevents or minimizes these toxic reactions (Abernathy and Spink 1952 Halberg *et al* 1956) Intramuscular injections of corticotropin in a dose of 40 mg every 8 hours or oral prednisone in dose of 20 mg 3 times daily, will help in causing the subsidence of toxemia and fever

Antibrucella vaccines have been used for therapy in countries other than the United States without agreement as to their value They are often avoided because of the dangers of creating conditions such as high antibody levels, which are favorable for accelerated lysis of the brucellae and consequently acute hypersensitivity reactions and vascular collapse

EPIDEMIOLOGY

Brucellae are transmitted to man from the natural reservoirs in domestic animals Data on insect transmission and the isolation of brucellae from wild rodents suggest the existence of secondary cycles which influence the natural history of human and animal infection Transmission to man occurs by contact ingestion accidental inoculation and inhalation (Reviewed by Renoux 1959) Contact with infected animals or their tissues or fluids allows brucellae to invade microscopic abrasions of the skin and the mucous membranes Ingestion of contaminated milk cream cheese or other dairy products made from raw milk containing brucellae is the second major source of infection Pickled meats and other uncooked foods contaminated by excretions of infected animals have also been incriminated Studies of laboratory acquired brucellosis of humans and animals suggest that invasion may occur via the respiratory route

Brucellosis is a significant occupational hazard among veterinarians meat packing house and rendering plant workers farmers butchers stock buyers stock handlers and laboratory workers (Reviewed by Sadler 1960) In humans the over all incidence is considerably higher in males than in females However the incidence is essentially the same in the two sexes where infection is due to use of unpasteurized dairy products indicating that differences where they occur are due not to unequal susceptibility of the sexes but to extent of exposure (Hardy *et al* 1931)

The geographic distribution of brucellosis has been presented in map and tabular form for Europe 1922 to 1955 by Wundt (1956) No European country except Denmark is free of the disease Human infection parallels animal infection and differences between adjacent areas are due to the differences in control of the animal disease Most

important it is suspected that inadequate performance of diagnostic procedures to detect brucellosis in domestic animals has led to the exportation of infected animals into otherwise clean areas

Swine brucellosis has remained localized because of its self limiting nature and be cause there is less opposition to slaughter of infected swine than to slaughter of other animals Wundt states that in Hungary and northern Rumania a focus exists which has progressed in single outbreaks into Austria and Yugoslavia (the latter country now hav ing almost eradicated the disease by rigorous measures) and has spread gradually over larger areas of Czechoslovakia The Alps separate Central Europe from the endemic area of melitensis infection in Southern Europe Migrating flocks of sheep bring it to the plains and along the coast (a method of spread also observed in the Middle Eastern countries such as Iran) Melitensis infection was spread into Western Germany after World War II by migrating flocks of sheep from France

Studies carried out on goats in the province of Cordoba Spain using the serum aggluti nation test the milk ring test and culture of milk sediment showed that about 16 to 29 per cent of 1 538 animals studied in 118 herds could be considered infected Of the 118 herds 111 contained infected animals (Elberg 1959) on the basis of having 40 International Agglutinating Units per ml of serum In Latin America the highest preva lence is among dairy animals swine and goats are second in incidence and sheep third (Szyfres *et al* 1959)

In the United States fewer than 600 human cases were reported in 1961 The disease has been maintained in large part by the per sistence of the infection in swine about 5 per cent of herds are infected Iowa Kansas and Illinois report the greatest number of cases Certain states according to Steele report more human cases than would have been predicted from the rate of elimination of bovine brucellosis they include Tennessee Georgia New York Nebraska South Dakota and Minnesota The remaining high incidence states are Virginia Arkansas Louisiana Texas and California Figures for human incidence are probably one fifth or less of the actual infections which occur in humans Diagnostic difficulties the biology of the disease which includes symptomless and mild unrecognized cases and finally poor report ing contribute to the unreliability of the reported figures

In the U S S R human brucellosis is most closely related to sheep brucellosis whereas bovine brucellosis is of no great importance as a source of human infection About 3 000 human cases occur annually in the U S S R about 15 per cent of the disease incidence in 1952 The improvement is due partly to the annual human immunization of 5 million persons o cupationally exposed and to the excellent but short term effectiveness of the massive immunization of 11 million sheep with strain 19 of *Br abortus* Dermal route revaccination of humans is practiced 2 to 3 months before the lambing season or before the main slaughter of sheep Where vac cination of sheep is not customary as in the Ukrainian Republic brucellosis has been controlled by the slaughter of entire flocks which contained reactors to the serologic tests

CONTROL

It is accepted in most of the western countries that the problem of human infection will be solved with the control of infection in the animal reservoir Measures to accom plish this are based on regulations governing interstate movement of infected animals a program designed to immunize young ani mals segregate and/or slaughter serologically positive animals improve environmental sani tation and educate the public concerning the disease With regard to serologic detection by the routine agglutination test the gradual decrease of the infection in certain areas means that reactor animals will become more difficult to find The application of the Coombs test for blocking type antibody plus the complement fixation test then may be used to improve the detection of weak reactor animals

RESISTANCE TO INFECTION

Epidemiologic data as well as experiments on human volunteers suggest that from 50 to 80 per cent of human beings are sus ceptible to infection by *Br melitensis* Under

severe exposure, 40 to 75 per cent of those infected exhibit either frank or abortive clinical attacks (Morales-Otero, 1929, Spink *et al*, 1962) In general, a lower proportion of *abortus* infections leads to clinically apparent disease than is the case with the *suis* and the *melitensis* infections In the United States, not more than 50 per cent of the human population is susceptible to *Br abortus* *These figures correspond to natural* outbreaks of the disease in limited groups acquiring the infection primarily from contaminated milk, it may be higher for other portals of entry

Young animals are more resistant to infection than adults with the exception of swine The great majority of human cases occur between the ages of 12 and 60 When the disease occurs in an undernourished population brucellae will infect all age groups and children respond as severely as adults (Bothwell 1962)

The existence of partial immunologic tolerance is suggested from studies on young lambs in which the newborns were exposed daily to *Br abortus* for the first 65 days of life When re-exposed to the infection as young adults the animals either failed to produce agglutinins or showed a lag phase as contrasted with the controls In the test animals the proportion of blocking type antibodies was increased Furthermore the agglutinins persisted for a shorter period in the test group However no effect was observed on the complement fixing antibodies (Nagy 1963)

So-called nonspecific factors are of major importance in susceptibility and recovery Macrophages derived from animals immunized against brucella are able to resist not only the cytotoxic action of brucella but also that of *Mycobacterium tuberculosis* (Elberg Schneider and Fong 1957) Similarly macrophages from animals vaccinated with BCG also resist the cytotoxic action of brucellae The cross resistance phenomenon has been observed in guinea pigs by Pullinger (1936) and Henderson *et al* (1956) and in the mouse by Sulitzeanu *et al* (1962) Hellman (1962) has extended the range of cross resistance in the guinea pig to include *Listeria monocytogenes* The general principle appears to involve cellular immunity and intracellular parasitism Thus far, only organisms which lead an intracellular existence in their host induce the kind of cross resistance described above The serum factor required for the demonstration of the macrophage immune reaction appears to be nonspecific a variety of unrelated antisera will potentiate cellular immunity equally well (Elberg *et al*, 1958)

Immunity following natural infection or vaccination with strains of attenuated virulence is relative in the sense that it can be overcome by strains of exceptional virulence or by massive exposures Evidence of reinfection in humans is available The development of immunity appears to occur in two phases The first or superinfection immunity phase (Pollack *et al*, 1952) occupies the time during which the original infecting organisms are still present in the tissues and during which the host shows a marked rise in resistance to reinfection The second or sterile phase covers that period after the disappearance of the organisms of the primary (immunizing) infection

The only clear-cut examples of a protective role of antibodies comes from the studies on monospecific sera and classic colostral immunity Sulitzeanu (1958) has shown a correlation between agglutinating activity of the serum and its ability to enhance the removal of the bacilli from the spleen

Many preparations have been tried for vaccination against brucellosis in cattle including virulent and avirulent living suspensions killed vaccines and fractions of cultures Repeated tests have proved that the strain 19 of *Br abortus* isolated by Buck in the United States in 1923 is an efficient immunizing agent when given subcutaneously intradermally or intracaudally It is not ordinarily excreted in the milk and its biologic properties are stable during field use It is highly agglutinogenic in the adult animal but when used during calfhood (Haring *et al* 1947) *the agglutinin re*sponse is temporary and eventually weakens sufficiently not to confuse later titers due to field infections Strain 19 is virulent when used on pregnant animals or when accidentally inoculated into man It may reduce milk yields temporarily and rarely may also be fatal to calves (Roberts *et al* 1962)

However it does reduce the abortion rate to a level of about 2 per cent (McDiarmid 1960)—a level perhaps due to other abortion inducing conditions About 80 per cent protection is induced by strain 19 against relatively light challenges and about 50 per cent protection against 10 fold higher challenge infections The calfhood protection induced by a single inoculation of strain 19 lasts until about the fifth pregnancy and possibly longer (Goode *et al* 1957)

In an attempt to avoid living vaccines or agglutinogenic preparations studies have been conducted with heat killed organisms and adjuvant substances (such as Falba in conjunction with mineral oil) using smooth mucoid and rough strains (Berman and Irwin 1954) *Br abortus* strain 45/20 gave almost as good protection in laboratory animals and cattle as strain 19 with the added advantage over the latter of being non agglutinogenic However the fact that it requires two doses to be effective against relatively mild infection (McDiarmid and Sutherland 1957 McEwen and Samuel 1955) is a disadvantage which far outweighs the relative efficacy of the preparations (Mc Diarmid 1961) The mucoid strain developed by Huddleson (1947) has not proved to be useful

Major advances have been made in the effort to produce effective immunizing agents for sheep and goats since 1957 mainly as a result of coordinated experimentation sponsored by the World Health Organization and the Food and Agriculture Organization of the United Nations Renoux (1959) utilized a formalin killed strain of *Br melitensis* mixed with Arlacel (mannide monooleate) and mineral oil Effective in protecting pregnant goats this vaccine had the disadvantage of causing persistent local reactions at the site of inoculation and prolonged serologic reactions An alternate line of approach (Herzberg and Elberg 1955) employed an attenuated streptomycin independent strain designated Rev I isolated from a streptomycin-dependent population of *Br melitensis* (earlier proved to be too attenuated for practical use) which was able to confer solid immunity on pregnant goats (Elberg and Faunce 1957) A comparison of two vaccines (Rev I *vs* adjuvant killed cells) revealed little difference as far as resistance to a 50 per cent infection dose but greater ability of Rev I to induce tissue clearance of the challenge infection (Jones *et al* 1958a b) The ability of Rev I vaccine to protect pregnant goats against natural exposure was confirmed in controlled experiments in Malta (Alton 1959 1961a b 1962) Iran (Jones *et al* 1963) and Israel (Neeman *et al* 1963)

The Rev I strain confers striking protection against brucella epididymitis in young rams Laboratory and field trials comparing strain 19 of *Br abortus* and Rev I demonstrated long lasting immunity to severe natural infection as a result of Rev I vaccination and relatively poor protection by strain 19 (van Heerden and Rensburg 1962 Van Drimmelen 1960) Mixtures of vaccine strains did not improve protection Solid and complete immunity was induced by the Rev I strain alone whereas only poor protection was conferred on yearling rams by killed *Br ovis* cells alone (Biberstein *et al* 1962) Further trials on pregnant sheep comparing strain 19 and Rev I have confirmed the solid immunity induced by the Rev I strain against severe natural infection (Jones *et al* 1963 Ivanov and Kirillov 1962)

Excretion of Rev I in the milk of occasional goats and sheep has been reported The deliberate serial passage of the vaccine strain by various routes through pregnant goats failed to bring about alterations in bacteriologic properties or virulence (Elberg and Faunce 1962) The strain is extremely stable in all of its characteristics and is readily differentiated from virulent field strains of *Br melitensis* by rate of growth and antibiotic sensitivity pattern It is agglutinogenic in all animals but may be distinguished by the complement fixation test from naturally infected animals If given to pregnant animals the vaccine may induce abortion when injected 35 days after mating When given before breeding it does not induce abortion (Elberg and Meyer 1958)

Other approaches toward immunization of sheep and goats include (1) vaccines containing a living smooth avirulent *Br abortus* strain 112 mixed with a killed culture of a virulent strain (Lafenetre and Carrere

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Many preparations have been tried for vaccination against brucellosis in cattle including virulent and avirulent living suspensions killed vaccines and fractions of cultures Repeated tests have proved that the strain 19 of *Br abortus* isolated by Buck in the United States in 1923 is an efficient immunizing agent when given subcutaneously intradermally or intracaudally It is not ordinarily excreted in the milk and its biologic properties are stable during field use It is highly agglutinogenic in the adult animal but when used during calfhood (Haring *et al* 1947) the agglutinin response is temporary and eventually weakens sufficiently not to confuse later titers due to field infections Strain 19 is virulent when used on pregnant animals or when accidentally inoculated into man It may reduce milk yields temporarily and rarely may also be fatal to calves (Roberts *et al* 1962)

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1956) and (2) attenuated strains such as strain BA 19 of *Br abortus* used in the USSR (Orlov 1960)

Human immunization of those occupationally exposed with a derivative of *Br abortus* strain *BA 19*, by the subcutaneous route has been carried out on a large scale in the USSR in view of the probability that control of the animal infections will not come for many years (Vershilova *et al* 1961) The massive vaccination campaign according to Vershilova and colleagues has resulted in a major reduction in the incidence of human brucellosis without undue risk of clinical complications due to immunization The report of Spink *et al* (1962) suggests a more conservative viewpoint of the safety of the Soviet strain BA 19 vaccine Despite such reservations the Soviet vaccine has received intensive field trials and is a licensed biologic product for use there The useful introduction of the dermal vaccination method was carried out by Smirnov *et al* (1958)

The Rev I and BA 19 strains were studied in humans by Spink *et al* (1962) Using 250 million cells of Rev I clinical disease was produced in 11 of 16 subjects Two of the 16 receiving BA 19 developed acute brucellosis Further studies carried out on cynomolgus monkeys have shown that as few as 260 cells of Rev I given subcutaneously induce a 1 000 to 10 000 fold increase in resistance over that of nonimmunized animals against respiratory challenge (Elberg and Faunce 1964)

Cell wall products have been tested by several groups for their immunizing effectiveness (Markenson *et al* 1959 Allen 1961 Smith *et al* 1962 Foster and Ribi 1962) Killed whole cell vaccines were prepared by ultrasonic inactivation by Gargani (1961) All of these preparations give encouraging results in the mouse and the guinea pig but further study is needed before their value against naturally acquired infection can be established

The use of immunizing mixtures made up of several bacterial species such as anthrax brucella tularemia and plague has led to controversial results Where the experiments have been carried out rigorously actual interference with immunity to brucella is characteristically the end result of the mixed prophylactic reagents (Borodko and Sanсonovich 1963)

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	Growth Factors				
	X	V	Iridescence	Capsules	Hemolysis
H influenzae					
Typable a f	+	+	+	+	0
Nontypable	+	+	0	0	0
H aegyptius	+	+	0	0	0
H parainfluenzae					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemolyticus	+	+	?	?	+
H parahemolyticus	0	+	?	?	+
H suis					
Typable	?	?	+	+	0
Nontypable	?	?	0	0	0
H parasuis					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemoglobinophilus	+	0	?	?	0

? = Not determined

forms known to be widely distributed in the normal nasopharynx Nor had methods then been developed for distinguishing the true influenza bacillus from closely allied species

However certain facts were learned from the extensive bacteriologic investigations carried out during the pandemic of 1918 Davis (1917 1924) Thjotta and Avery (1921) Fildes (1921) and Rivers and Poole (1921) extended our knowledge of bacterial growth factors and standardized procedures for the use of X and V factor requirements as a diagnostic aid In brief they showed that whole blood contained both factors Their action could be separated by exposing whole blood extracts to 250 F V factor was thus destroyed A yeast extract sterilized by filtration served as a good source of V factor

A study of the nutritional requirements of strains diagnosed as influenza bacilli during the pandemic of 1918 led to the discovery of some new organisms Pritchett and Stillman (1919) reported an organism which they labeled X bacillus a beta type of hemolysis appeared following the growth of this organism on blood agar and X factor was not needed for growth Rivers (1922) described strains which he named *B parainfluenzae* they differed from true *H influenzae* only in their ability to grow in the absence of X factor Hemolytic strains requiring both X and V factors have been reported by both Fildes (1924) and Valentine and Rivers (1927)

Search for evidence of a filtrable virus in patients with influenza also met with failure as outlined by Jordan (1927) and Scott (1929) Technics for the isolation and the identification of viruses were just beginning to be explored It is believed that the choice of patients too late in their disease to yield the virus and the use of immune individuals as recipients were responsible for the failure to transmit the virus to human beings Nonetheless the view was held by a number of investigators of the 1918 pandemic that its unprecedented severity reflected the concurrent interplay of a virus and the influenza bacillus This thesis was strengthened by the recovery of a virus from swine influenza (Shope 1931) and by the demonstration that the synergistic effect of an influenza bacillus *H suis* and swine influenza virus is essential for both the natural and the experimental disease The importance of this contribution deserves emphasis it illustrates the enhancement of injury caused by the combined effect of a bacterial and a viral infection

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29

The Hemophilus Group

HEMOPHILUS INFLUENZAE

Hemophilus influenzae the type species of the hemophilus group has played two important roles in human infections (1) a secondary role in pandemic virus influenza and (2) a primary role producing pyogenic infections the latter occur infrequently in adults in children on the other hand *H influenzae* is one of the more frequent causes of serious pyogenic infections

The members of the hemophilus group are gram negative nonmotile nonsporebearing aerobic bacilli and lead a strictly parasitic existence they possess poorly developed enzyme systems therefore all of them require enriched media Their nutritional needs yet imperfectly defined display certain differences within the genus which have a limited but useful application in identification The hemophilic bacteria have been classified as in Table 1 on the basis of their needs for two growth factors X and V and other biologic traits

Role of *H Influenzae* in Pandemic Influenza

During the pandemic of influenza of 1890 Pfeiffer (1892, 1893) isolated from the nasopharynx of most of those suffering from the disease small straight gram negative bacilli tending to occur in clumps they stained with difficulty by ordinary dyes Loeffler s methylene blue revealed polar granules Growth in pure culture required substances present in whole blood, the growth stimulating substances were associated with the iron-containing portion of hemoglobin Blood agar plates sparsely seeded yielded small transparent colonies which produced no change in the surrounding medium

The frequency of occurrence of this organism in patients with influenza and its alleged virtual absence in normal individuals led to the erroneous conclusion that *H influenzae* was the cause of the influenza pandemic of 1890 thus this organism was named the influenza bacillus and in 1923 was designated *H influenzae* by The American Society of Bacteriologists Those who have objected to this terminology have continued to use the name Pfeiffer s bacillus

Investigations on the role of this organism in epidemics from 1892 to 1920 are reviewed in Kristensen (1922) Scott (1929) and Jordan (1927) The results cast doubt on the primary agency of the influenza bacillus in pandemic influenza

During the 1918 influenza pandemic extensive bacteriolgic investigations were carried out to determine the role of the influenza bacillus The results are reviewed by Jordan (1927) and Scott (1929) There is no doubt that most investigators who studied this special problem and therefore had special interest in looking for *H influenzae* found a very high incidence not only in the nasopharynx but also in postmortem lung cultures Unfortunately the methods then available could not differentiate between the encapsulated and thus potentially pathogenic influenza bacilli and the nonencapsulated

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	GROWTH FACTORS				
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Nontypable	+	+	0	0	0
H aegyptius	+	+	0	0	0
H parainfluenzae					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemolyticus	+	+	?	?	+
H parahemolyticus	0	+	?	?	+
H suis					
Typable	?	?	+	+	0
Nontypable	?	?	0	0	0
H parasuis					
Typable	0	+	+	+	0
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H hemoglobinophilus	+	0	?	?	0

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forms known to be widely distributed in the normal nasopharynx Nor had methods then been developed for distinguishing the true influenza bacillus from closely allied species

However certain facts were learned from the extensive bacteriologic investigations carried out during the pandemic of 1918 Davis (1917 1924) Thjotta and Avery (1921) Fildes (1921) and Rivers and Poole (1921) extended our knowledge of bacterial growth factors and standardized procedures for the use of X and V factor requirements as a diagnostic aid In brief they showed that whole blood contained both factors Their action could be separated by exposing whole blood extracts to 250 F V factor was thus destroyed A yeast extract sterilized by filtration served as a good source of V factor

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The investigations of Shope (1931 1944) raise the question whether the facts disclosed for swine influenza also hold true for human pandemic influenza Since the swine epidem

ics appeared for the first time concurrently with the human influenza pandemic of 1918 Shope suggests that the latter disease originated in swine A study of the epidemiology of the swine disease demonstrated that the virus lies dormant in lung worms which appear to live in symbiotic relationship in the swine lungs Under appropriate climatic conditions *H suis* is found in increasing numbers in the nasopharynx of the experimental animals The virus ceases to lie dormant and epidemics of swine influenza are launched

The clinical and pathologic similarities of swine and human influenza have led several investigators to explore the synergistic action of human influenza viruses and *H influenzae* the conflicting results have been reviewed and extended by Bang (1943)

The effect of *H influenzae* on both human and swine virus deserves re-examination in light of current concepts concerning the biology of *H influenzae* However it is evident from our present knowledge that the human and the swine influenza viruses as well as the two varieties of *Hemophilus influenzae* and *suis* possess fundamental differences Even if it could be shown that the swine virus had been the primary cause of the 1918 human pandemic subsequent epidemics of influenza have clearly been the results of different viruses Evaluation of the importance of synergism between an influenza virus and a bacillus in determining the severity of human pandemics must await additional study

The contributions of Pittman (1931) clarified some of the controversial issues Strains of *H influenzae* prevalent in the healthy human respiratory tract were shown to differ from those isolated from persons with *H influenzae* infections It was confirmed that strains cultivated from patients with meningitis bacteremia and pneumonia could be differentiated from nonpathogenic forms by their iridescent growth on Levinthal agar capsules could be demonstrated by special technics Six different types of *H influenzae* were identified by precipitation and agglutination tests the former method demonstrated the presence of the specific soluble substance by its precipitation with homologous diagnostic typing antiserum The 6 types were designated *a b c*, *d e* and *f* type *a* specific substance was shown to be a polysaccharide Virtually all of the meningitic strains were type *b* Dried alcoholic precipitates of the cultures of 2 of the strains reported by Rivers (1921) were also identified as type *b*

Role of *H Influenzae* As a Primary Pyogenic Agent

Clinical Patterns The important role of *H influenzae* in pyogenic disease is well documented in infants and children this organism is one of the most frequent causes of serious bacterial infection The clinical patterns in which type *b H influenzae* plays an important role are described below

Upper Respiratory Tract Infection Nasopharyngeal infection can be shown to precede all the clinical patterns of localized *H influenzae* pyogenic infection for example, meningitis pyarthrosis epiglottitis as well as pneumonia sinusitis and otitis media There is reason to believe that most patients are febrile initially but recover spontaneously without serious disease as a result of their immune response The clinical features of *H influenzae* nasopharyngitis cannot be distinguished from those caused by other frequently occurring bacterial infections for example in patients suffering from group A hemolytic streptococcus or pneumococcus infections Moreover the simple laboratory procedures total white blood cell count and erythrocyte sedimentation rate show similar responses in all of these varieties of pyogenic bacterial infections In view of the widespread custom of using penicillin alone as treatment for patients sick with respiratory infections of unknown etiology it is important to emphasize that *H influenzae* is not susceptible to this antibiotic in the dosage in general use Treatment will be discussed later

Pediatricians are very aware of the potentiality of *H influenzae* for invasion of the blood during nasopharyngitis and the possibility of subsequent localization in a number of areas with development of clinical patterns to be described We have also seen spontaneous subsidence of bacteremia without localization

Patterns Caused by Direct Spread in the Respiratory Tract *Otitis Media* The frequency of *H influenzae* otitis media in childhood was reported by Mortimer and

Watterson in 1956 about one third of their patients showed *H influenzae* when examined carefully for the etiologic agent There are no features which distinguish *H influenzae* otitis media from other varieties Examination of exudate when the tympanic membrane ruptures spontaneously is clearly indicated for etiologic diagnosis and best treatment A method providing immediate identification of typable *hemophilus* will be described under Identification

Sinusitis Maxillary sinusitis appears to be frequent following type *b H influenzae* nasopharyngitis and may persist over many months in a chronic form when untreated However infection of the ethmoid sinuses is characteristically very acute Nowadays older infants and children who after a short febrile illness suddenly develop in either one or both eyes periorbital cellulitis with edema which closes the eye are most likely to have *H influenzae* ethmoiditis Confirmation of type *b H influenzae* as the etiologic agent when found in the respiratory tract is obtained by growth of the organism from the blood in most patients

Pneumonia Clinically *H influenzae* pneumonia can not be differentiated from pneumococcus pneumonia There is reason to believe that bacteremia is more frequent in the former When growth occurs in the blood cultures of patients with pneumonia the etiology is clearly defined When type *b H influenzae* is identified only in cultures of the nasopharynx in a patient with pyogenic pneumonia in the absence of other bacterial pathogens its etiologic role in the pneumonia is further supported by the patient's agglutinin response within 2 weeks In young infants *H influenzae* pneumonia is usually associated with bacteremia and frequently with empyema the frequency of meningitis in association with empyema and the difficulty in detecting meningeal inflammation by the usual clinical signs lead most pediatricians to examine the spinal fluid of young infants with *H influenzae* pneumonia and empyema

Epiglottitis The only clinical pattern caused by *H influenzae* localization associated with bacteremia in which the clinical signs are pathognomonic for this infectious agent is epiglottitis or obstructive laryngitis There is an age predilection virtually all patients are older than 2 years The first report of this clinical syndrome by Lemierre *et al* (1936) described adults with this disease Characteristically dyspnea develops suddenly after signs of a mild respiratory infection with little fever progression of obstruction is so rapid that most patients are admitted to the hospital within several hours of onset requiring tracheotomy for relief of obstruction Constitutional signs of overwhelming infection develop at a comparable rate In spite of these alarming signs phonation is efficient On physical examination the only other constant sign found is the one which has proved to be pathognomonic of *hemophilus* obstructive laryngitis an enlarged misshapen epiglottis easily seen when the tongue is depressed In patients who exhibit these signs immediate relief of obstruction by tracheotomy is mandatory The risk of sudden death from respiratory and cardiac arrest is so great that most pediatricians prepare for tracheotomy before examining the epiglottis Specimens of nasopharyngeal mucus and blood are obtained for culture only after tracheotomy The signs of overwhelming infection are explained by the presence of bacteremia in virtually all untreated patients Direct laryngoscopy shows the obstruction to be entirely upraglottic the competence of phonation is thus explained These patients are hospitalized for relief of obstruction so soon after onset that there is not time for the development of purulent foci Since the development of a number of effective therapeutic agents against *H influenzae* we have not seen a single patient with any additional localized infection

Meningitis is a frequent consequence of *H influenzae* bacteremia In infants and children type *b H influenzae* is the commonest cause of meningitis The first authentic case of influenzal meningitis was reported in 1899 The importance of this organism as a cause of meningitis was emphasized by Rivers in 1922 Prior to 1938 the mortality from this disease was virtually 100 per cent

There are no clinical signs which distinguish *hemophilus* from other varieties of pyogenic bacterial meningitis The findings on physical examination depend more on the

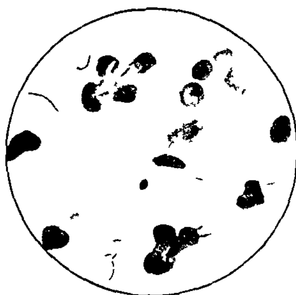


Fig 1 Gram stain of purulent spinal fluid infected with *H influenzae* type b × 800

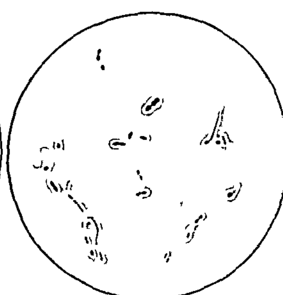


Fig 2 Typing of *H influenzae* by capsular swelling with diagnostic typing sera × 1050 (Alexander H E 1934 Treatment of Haemophilus influenzae infections and of meningococcus and pneumococcic meningitis American Journal of Diseases of Children 66 172 187)

age of the patient the duration and the severity of infection and whether some specific treatment has been given than on the bacterial agent which causes meningitis Examination of spinal fluid provides immediate information in most patients When pyogenic bacterial infection is present the cellular response is almost entirely polymorphonuclear and the concentration of sugar reflects the degree of severity the lower the concentration of sugar the more severe the infection In most patients a Gram stain of the spinal fluid directly or of sediment after concentration will reveal organisms When gram negative bacilli of the morphology shown in Figure 1 are seen immediate identification can be made by demonstration of capsular swelling by type specific antibody when *H influenzae* is the cause of the infection, as shown in Figure 2 Details of these examinations will be described later

The evolution of the meningeal infection during the first 24 hours prior to treatment varies greatly Most patients seen within 24 hours of onset exhibit signs of relatively mild meningitis Only signs of meningeal inflammation are seen the sensorium is clear There are no signs of generalized encephalitis or of localized cerebral cell damage The spinal fluid findings are consistent with mild meningitis—few organisms and little if any reduction in the spinal fluid sugar These patients recover completely under optimal therapy

A significant number of patients have already developed serious disease by the time they are seen Even though there are a variety of clinical patterns in this group with early severe disease they all appear to have in common clinical signs of encephalitis Marked depression of the sensorium is characteristic of almost all The extent and the reversibility of the encephalitic lesions determine whether these patients recover completely survive but have persistent cerebral cell damage or die despite all measures known A particularly fulminating form of central nervous system disease was seen during 1957 when Asian influenza was widespread The disease was characterized by sudden onset of respiratory failure at times before it was recognized that meningitis was present Several patients followed similar courses type *b H influenzae* was isolated from all All patients died despite heroic therapeutic measures The occurrence of this

syndrome during the Asian influenza epidemic suggests a possible influence of the virus disease on the host parasite relationship of the *hemophilus* infection. A description of clinical features can be found in Cooke (1964).

Pyarthrosis and Osteomyelitis. During the past decade, when all bacterial infection of the bones and the joints has greatly decreased in incidence, *H. influenzae* has been one of the more commonly occurring varieties. Most patients show primarily a pyarthrosis; when involvement of bone can be demonstrated, it is usually an epiphysitis. Osteomyelitis is rare. The joint inflammation occurs in the large joints, characteristically most commonly the elbow and the knee. In infants, hip joint pyarthrosis is difficult to diagnose; the lesion is frequently far advanced and the damage irreversible. Bacteremia is demonstrable in most patients. We have been impressed by persistence of joint swelling and tenderness along with fever up to 3 weeks, even though the organism has been eliminated by effective treatment after 1 week.

NONTYPABLE *H. influenzae* AND OTHER INFLUENZA BACILLI. It is seldom possible to assign a primary pathogenic role to nonencapsulated *H. influenzae* in any age group. In young infants, meningitis and pneumonia accompanied by bacteremia are occasionally caused by these organisms. Epidemic conjunctivitis has been ascribed to an organism labeled previously "Koch-Weeks bacillus," a member of the influenza bacillus group (Koch, 1887, and Weeks, 1887), but now classified *H. aegyptius*; it is indistinguishable by ordinary criteria from nontypable *H. influenzae*. Recent genetic studies suggest that *H. aegyptius* is a variant of *H. influenzae* (Leidy, 1959). Subacute bacterial endocarditis associated with brain abscess at times is caused by *H. influenzae* and *H. parainfluenzae*, both in children and adults (Rose, 1941). The role which this organism plays in chronic lung infections and in some acute lung infections, when the blood cultures are sterile and no other bacterial pathogens are demonstrable, is controversial. According to some opinions, the influenza bacillus has a destructive action on bronchial epithelium (Zinneman, 1943).

IDENTIFICATION OF HEMOPHILUS SPECIES

When organisms are sufficiently numerous in biologic fluids to be seen on stained smear, species and type may be identified by capsular swelling (Fig. 2) or precipitin test within a few minutes. The capsular swelling test is the simpler procedure.

A 3 mm. loopful of diagnostic typing antiserum is mixed on a cover slip with an equal quantity of pathologic fluid. Enough methylene blue is added to color the drop lightly, and the preparation is inverted on a hollow ground slide and sealed with oil. The cover slip may be inverted on a flat slide.

The precipitin test can also provide immediate diagnosis when organisms are numerous; when the infection is so mild that bacteria cannot be demonstrated microscopically, this procedure is seldom positive. The technic is as follows:

In a small precipitin tube (50 × 6 mm.), a 1-cm. column of fluid to be tested is carefully layered by a fine capillary pipette on an equal column of diagnostic rabbit antiserum. A precipitate in the form of a white ring at the interface represents a positive test. The antiserum used and the pathologic fluid must be perfectly clear. Speed of formation of the ring varies with the concentration of specific soluble substance in the fluid; immediate appearance of the ring denotes high concentration. This time factor may be used as an index of severity of infection of the blood or the spinal fluid.

These procedures may be used for immediate diagnosis of *H. influenzae* in spinal fluid, middle ear or joint exudate, or empyema fluid; a concentrated suspension of nasopharyngeal mucus from patients with obstructive laryngitis or pneumonia due to this organism may reveal its presence when swelling of the capsule is demonstrated. The specimen of mucus is collected on a small cotton swab (Alexander *et al.*, 1941) passed through the nares to the posterior pharyngeal wall, where it is allowed to remain for several seconds to collect mucus; at the bedside the swab is placed in a small tube containing 0.2 ml. of sterile broth.

Identification of encapsulated *H. influenzae* in cultures from the blood or other fluid may be made after incubation, usually for 18 hours, by capsular swelling with

type specific diagnostic antiserum When the latter test is negative Levinthal agar is inoculated to test for iridescence of growth The importance of a gram stained smear deserves emphasis otherwise serologic crossing between certain types of *H influenzae* and pneumococci will lead to errors in diagnosis (See Antigenic Structure)

If neither capsular swelling nor iridescent quality of growth is demonstrable diagnosis of *H influenzae* must depend on requirements for both X (hemin) and V (diphosphopyridine nucleotide) factors for growth

Classification of Influenza Bacillus Group According to X and V Factor Requirements

REQUIRE X AND V FACTORS FOR GROWTH

1 *H influenzae*

A Typable potentially pathogenic strains are encapsulated They are classifiable into 6 specific types by swelling of the capsule or precipitation of the soluble specific substance by specific antibody A characteristic iridescent growth is produced on Levinthal agar

B Nontypable noniridescent nonencapsulated seldom pathogenic organisms cannot be differentiated from encapsulated *H influenzae* on blood agar by morphology of individual members or their colonies Moreover they also require X and V factors Their failure to produce iridescent growth on Levinthal agar identifies them as nontypable nonencapsulated variety

2 *H hemolyticus* Production of beta hemolysis in blood agar distinguishes this organism from *H influenzae* Except for its rare occurrence as a cause of subacute bacterial endocarditis no pathogenic role is recognized It is found frequently in the normal nasopharynx

V FACTOR BUT NOT X IS REQUIRED FOR GROWTH

1 *H parainfluenzae* Human pathogenicity appears to be limited to subacute bacterial endocarditis It is considered as a normal inhabitant of the human nasopharynx Individual organisms are morphologically similar to *H influenzae* except for greater regularity of form and less evidence of autolysis Colonies on blood agar are indistinguishable from those of *H influenzae* This class includes two groups

A Encapsulated *H parainfluenzae* (Lenert and Alexander unpublished) Capsular swelling can be demonstrated with type specific rabbit antiserum and iridescent growth can be demonstrated on Levinthal agar There appears to be more than one type

B Nonencapsulated *H parainfluenzae* Growth is noniridescent, and capsular swelling cannot be demonstrated

2 *H parahemolyticus* Production of beta hemolysis distinguishes this organism from *H parainfluenzae* Human pathogenicity is not unlike that described for *H hemolyticus* which requires both X and V factors

H suis Essential growth needs have not been defined This organism reacts synergistically with the virus of swine influenza in the natural and experimental disease of hogs Human pathogenicity is unknown Morphologically this organism does not differ significantly from *H influenzae* and *H parainfluenzae* While Levinthal broth and chocolate agar provide better growth than blood agar growth of most strains on these media is poor Shope reported that X and V factors are essential for growth but study of a number of strains obtained from Dr Shope shows that some do not require X factor In our experience the best growth has been obtained on a modified Levinthal agar with the addition of 5 per cent horse plasma or 10 per cent yeast extract (fresh)

Study of 8 strains revealed 2 different groups

1 Those producing iridescent growth on modified Levinthal medium Capsular swelling occurs on exposure to homologous rabbit antibody Use of antisera produced against 3 iridescent strains failed to reveal immunologic differences among 5 strains No immunologic relationship was demonstrated between the type specific antigens of *H suis* and *H influenzae*

2 Those showing noniridescent growth demonstrated no type specific characteristics

REQUIRE X FACTOR AND NOT V

H hemoglobinophilus is the only known representative of this group Human pathogenicity has not been recognized Friedberger (1903) first described *H hemoglobinophilus* in chronic purulent exudate from the preputial sac in dogs

Examination of X and V Factor Requirements of Unknown Organisms For this purpose media must be available for testing

the separate and combined action of X and V factors

X FACTOR MEDIUM Equal parts of Levinthal stock (used in Levinthal agar) autoclaved 15 minutes at 20 pounds pressure and melted Proteose Agar No 3 (Difco) (45 Gm per liter plus Bacto Agar 15 Gm per liter) X factor is stable and can withstand this treatment V factor is destroyed

V FACTOR MEDIUM One part of yeast extract to 9 parts of melted Proteose No 3 Agar (Difco) Yeast extract can be prepared by the following method modified from Thjotta and Avery (1921) Emulsify 100 Gm of powdered brewer s yeast in 400 ml of distilled water Adjust pH to about 4 6 boil for 10 minutes Filter emulsion through filter paper adjust filtrate to pH 7 0 and filter through Seitz filter to sterilize Transfer to a sterile container fitted with a glass stopper and seal with sterile petroleum jelly

COMBINED X AND V FACTORS One part of yeast extract to 9 parts of X factor medium

Media are distributed in 3 ml quantities in tubes (100 × 13 mm) and slanted

Cultures to be tested are grown on Levinthal agar a loopful is suspended in 0 2 ml physiologic saline just before inoculating the separate factor media

Pure hemin and coenzyme I may also be used as X and V factors

Pickett and Stewart (1953) report that *H influenzae* and *H parainfluenzae* can be distinguished by their satellite formation about growth of catalase positive and catalase negative organisms

Table 1 shows the differential characteristics of the influenza bacillus group

As will be discussed later results of transformation studies using DNA from hemophilus strains may provide a genetic criterion for classification

Cultivation and Biochemical Characteristics

Growth from pathologic fluids may be obtained on blood agar or broth at pH 7 6 somewhat better on chocolate agar Optimal growth takes place in media in which the contents of the red cells are liberated either by heat as in Levinthal (1922) or by peptic digestion as in Fildes (1920) Both of these media have the additional advantage of transparency and therefore are more suitable for the study of the characteristics of individual colonies The presence or the absence of iridescence can be studied also by viewing the growth on the surface of Levinthal or Fildes agar in obliquely transmitted light Growth on these media is influenced by pH and availability of oxygen The optimal pH is 7 6 Increased aeration by frequent agitation or by use of shallow layers of broth enhances growth

The broth we have found most satisfactory is a further modification of the Pittman (1931) changes in Levinthal broth It is made by mixing 1 part of Levinthal stock with 3 parts of neopeptone broth (Lenert and Hobby 1947) Levinthal stock is prepared as follows brain heart infusion broth (Difco) made according to directions on the bottle is heated to vigorous boiling and sterile defibrinated horse blood is added to make a final concentration of 10 per cent The mixture is filtered through Whatman filter paper No 12 and the clear filtrate is sterilized by Seitz filtration

Levinthal agar is made by adding 1 part of sterile Levinthal stock to 1 part of melted agar (45 Gm Proteose agar No 3 (Difco) plus 15 Gm Bacto agar per liter of water)

Khristensen (1922) reported that *H influenzae* produced acid from proteins He suggested that this fact might be responsible for the diversity of opinion concerning the ability of this organism to ferment carbohydrates In his opinion evidence for fermentation of carbohydrates was lacking There is general agreement in any event that this function is of no differential value

Most strains of *H influenzae* produce indole This is true of a larger fraction of encapsulated strains than of the nonencapsulated There are strains in each group which show no indole production it has proved to be too variable a characteristic to aid in the classification of these organisms

One of the most consistent characteristics of *H influenzae* is its ability to reduce nitrates to nitrites Hoagland (1942) used quantitation of this action for measuring growth of *H influenzae*

The solubility of *H influenzae* in bile first described by Sellards and Sturm (1919) and confirmed by Pittman (1931) offers another

point of similarity to pneumococci This trait is characteristic of both pathogenic and non pathogenic varieties of *H influenzae* and therefore is of no differential value

Morphology

Any description of morphology must be related to the source of the organisms In pathologic fluids spinal (Fig 1) synovial or pleural the organisms are usually pre dominantly coccobacillary simulating diplo cocci an erroneous diagnosis of pneumo coccus is often made when Gram staining is unsatisfactory At times the bacilli occur in short chains and are so short that they are mistaken for streptococci Along with these forms it is virtually always possible to find definite bacilli some quite long at one end of some there is seen a spherical body stained only at the periphery Occasionally, the pre dominant shapes are very bizarre long slen der forms occurring together with thick bacilli which assume the contour of a club an elbow or other irregular outlines The possibility that these are variants which favor the emergence of a rough strain is suggested but demonstration of their capsules makes such an interpretation unlikely

In cultures the composition of the medium and the age of the culture determine to a great extent the morphology of *H influenzae* When Levinthal agar is seeded with 0 5 ml of Levinthal broth culture and incubated 2 to 4 hours most of the organisms are clearly bacillus shaped There are also thick forms irregular in outline as if the protoplasm were distributed irregularly and chain for- mation is common After 6 to 8 hours in cubation the short bacilli and the coccobacilli predominate and the long forms are in the minority regularity of morphology is char acteristic Cultures which have been growing for 24 hours contain a large amount of amorphous debris and the predominant rec ognizable form is the minute short poorly stained coccobacillus giving the impression that only a part of the organism takes the stain Evidence of autolysis becomes in creasingly apparent after 12 hours At first the organisms take the stain less readily later amorphous debris is prominent indi cating that the organisms have disintegrated Inoculation of a Levinthal agar plate with 0 5 ml of an 18 hour Levinthal broth cul ture results in growth in 3 to 4 hours Irides cence is visible by obliquely transmitted light within 4 to 6 hours, this quality becomes more striking during the next 2 hours and subsequently starts to decrease After 24 hours the iridescent quality is absent Paral leling this phenomenon the capsules disap pear, and the organisms disintegrate There is reason to believe that these 3 changes which occur simultaneously are the result of liberation of enzymes by the bacteria When a much smaller inoculum is used to seed Levinthal agar (a 2 mm loop of 18 hour Levinthal broth culture) maximum irides cence is seen in 18 hours the capsules are well preserved and evidence of autolysis of organisms is absent at that time Apparently a longer period is required for this smaller population to produce sufficient autolyzing enzymes In Levinthal broth the changes in morphology are similar but less pleomor phism is seen and autolysis proceeds more slowly

Antigenic Structure

The antigenic pattern of encapsulated pathogenic varieties of *H influenzae*, types *a b c d e* and *f* is determined by specific soluble substances produced by each type and concentrated in the capsule Goebel (reported by Pittman 1931) concluded that type *a* specific substance is a polysaccharide Dingle and Fothergill (1939) reported the polysaccharide nature of type *b* specific sub stance MacPherson *et al* (1946) agreed on the polysaccharide nature of types *a* and *b* and reported this to be true for types *c d* and *f* as well The substance responsible for type specificity in types *a b c* and *f* has been shown by Zamenhof and Leidy (1954) to be a polysugarphosphate Type *b* sub stance is a polyribophosphate (Zamenhof *et al* 1953) In 2 types *d* and *e* the type specific substances are polysaccharides which do not contain phosphorus or sulfur Only type *b* a polyribophosphate contains a pen tose as its sugar moiety (Zamenhof *et al* 1953) the other type specific substances contain hexoses or hexosamines (Rosenberg *et al* 1961) The type of a given strain of *H influenzae* may be established by capsular swelling agglutination of organisms of pre

cipitation of the specific soluble substance with diagnostic typing serum The type specific antigen is responsible for these 3 reactions and also for stimulating the production of protective antibody as well

It is of great interest that 3 types of *H influenzae* are related immunologically to certain types of pneumococci (Chapman and Osborne 1942 Alexander *et al* 1946) Table 2 shows the cross reactions as evidenced by capsular swelling between the type specific antigens of *H influenzae* and pneumococci

The characteristics of the somatic antigens are less well known Platt (1939) isolated 2 proteins a P substance making up the mass of the protein which requires destruction of the organism for its liberation and an M substance which is labile small in amount and apparently a surface antigen since it is freed from the intact organism by washing with saline The M substance is toxic for animals and is common to all strains whereas P substance is nontoxic and differs among strains Dubos (1941) obtained from an R derivative of type *b H influenzae* which had high toxigenic power an antigen lethal for rabbits in 0 1 mg doses immunization of rabbits resulted in resistance to 50 M L D of this substance The toxic substance is believed to be an endotoxin

Studies on the somatic antigens of the influenza bacillus group by immunologic procedures suggest the presence of a common antigen in all types of encapsulated *H influenzae* and in some nontypable varieties However recent investigations using transformation technics suggest important biologic differences

The agglutination reaction when incubated at 37° C for 2 hours showed the same degree of specificity as the precipitation test However when agglutination was carried out at 55 C for 4 hours (the conventional procedure) there were marked cross reactions among the different types This fact probably explains the failure of the agglutination test, in the hands of earlier observers to identify specific types which must have been present in a part of the population More recent studies have shown that the somatic antigens of all 6 types as well as some nonencapsulated strains exhibit immunologic cross reactions Exposure to the higher temperature apparently releases some somatic components The labile capsules of *H influenzae* cannot be identified after such treatment Pittman s study of variation of colonial forms of *H influenzae* demonstrated the process whereby an S strain under artificial cultivation becomes rough the changes observed explained earlier controversies on morphology and some of the failures to identify specific types It is apparent as emphasized by Pittman that the immunologic behavior of the influenza bacillus parallels in a number of respects that of the pneumococcus

TABLE 2 CROSS REACTIONS BETWEEN POLYSACCAHRIDES OF PNEUMOCOCCI AND TYPE SPECIFIC ANTIGENS OF INFLUENZA BACILLI*

PNEUMOCOCCUS	*H influenzae*
6 Sub group	Type a
6 Sub group	Type b
11 †	Type c
15 A †	Type b
29 Sub group	Type b
35 B †	Type b

* Alexander H E Leidy G and MacPherson C 1946 Production of types a b c d e and f *H influenzae* antibody for diagnostic and therapeutic purposes Journal of Immunology 54 207

† Not hitherto recorded

As mentioned earlier encapsulated forms of *H influenzae* can be differentiated into types by capsular swelling (Alexander 1939) This procedure is also useful for detecting changes in the state of the capsule Study of type *b H influenzae* through all phases of the growth cycle in Levinthal broth and agar reveals that the capsules are much more labile than those of pneumococci When large inocula are grown for 7 hours they begin to show deterioration and in 24 hours it is difficult to identify them Degeneration of the capsules is more delayed when the inoculum is small for this reason the vaccine used to produce rabbit antibody is prepared from 6 hour cultures of *H influenzae* on Levinthal agar The organisms if washed from the plate with 0 5 per cent formalinized saline and immediately iced exhibit good preservation of capsules for as

long as a few weeks However degeneration of capsules occurs even in the presence of 0 5 per cent formalin if the suspensions are allowed to stand at room temperature for as short a period as 2 hours

Antibody to *H influenzae* can be measured quantitatively in rabbit antiserum (*Alexander and Heidelberger 1940*)

Toxins and Host Range

A number of authors (*Jordan 1927* and Scott 1929) working with strains of *H influenzae* cultivated from patients with influenza during the pandemic of 1918 reported that the injection of some strains into animals was followed by lethal toxic injury However the size of the lethal dose suggests that the toxic effect was due to an endotoxin and not an exotoxin

There is no evidence that *H influenzae* produces a true exotoxin On the other hand the injurious effect caused by what are probably endotoxins may play a significant role in the pathogenesis of severe infections Whether or not the different pathologic potentialities described for some strains depend on their capacity to produce this material cannot be answered Nor do we have any evidence that antibody to these toxic substances is important in recovery from *H influenzae* infections On the other hand the antigenic importance of the toxic fractions isolated by Platt (1939) and Dubos (1941) warrants further exploration

H influenzae is not naturally pathogenic for any of the smaller animals Multiplication with invasion of the blood does occur in mice injected intraperitoneally with organisms suspended in mucin (Fothergill *et al* 1937) or when suspensions in brain are introduced intracerebrally (De Torregrossa and Francis 1941) These tests serve a useful purpose for testing efficacy of antibacterial agents but are not adequate for differentiation between pathogenic and nonpathogenic strains

In monkeys Blake and Cecil (1920) reported bronchiolitis and hemorrhagic bronchopneumonia following intratracheal introduction of a culture of a pathogenic influenza bacillus Wollstein (1911) produced meningitis in monkeys by intrathecal inoculation of *H influenzae*

Wright and Ward (1932) described an *in vitro* test which could distinguish between strains cultivated from spinal fluid and those found in the respiratory tract of many normal subjects the former were uninfluenced by normal rabbit blood the latter were killed All strains which were well preserved in rabbit blood produced a type specific soluble substance

Fothergill *et al* (1937) provided an *in vivo* method for evaluating the influence of some therapeutic agents on this organism In our experience (Alexander and Leidy 1943a) 2 to 200 organisms of type *b H influenzae* strains isolated from patients with *H influenzae* infections when suspended in mucin as first described by Miller (1933) are lethal for at least 50 per cent of mice infected by the intraperitoneal route Mouse protection tests can be used to check the validity of the quantitative chemical methods for determining potency of type *b* rabbit antiserum The protective element in the antiserum is polyribose phosphate antibody (Alexander *et al* 1944)

Pathogenesis and Immunity

Even though there is a significant incidence of *H influenzae* meningitis pneumonia and epiglottitis in adults which is not generally appreciated hemophilus infections are a major problem only in the pediatric age group The report of Ward and Fothergill in 1931 suggesting that this difference is the result of the bactericidal property of the blood in most adults—a property which is lacking in most infants and children—has been generally accepted as the explanation for this difference It is true that the age incidence of *H influenzae* meningitis seems to correlate with bactericidal power of blood according to tests carried out in small samples of individuals The protective factor in specific antiserum (Alexander *et al* 1942 1944) is antibody against the specific soluble substance this antibody apparently promotes phagocytosis *In other words* the defense mechanisms against hemophilus infections show a striking similarity to those effective against pneumococci

Recent knowledge on the role of complement in inflammation in general (Lepow 1960) suggests its participation in a non

specific way in the defenses against *H influenzae* also even though complement induced bacteriolysis may not be the specific mechanism of action

Of equal interest in the fields of pathogenesis and immunity are the factors which determine whether a child after invasion by *H influenzae* develops only a febrile upper respiratory infection or a severe disease A chronic respiratory infection with typable *H influenzae* may persist in some children for several months without emergence of a severe infection It is known that children develop type specific agglutinins within about 2 weeks after such an experience There is reason to believe that antibody response to typable *H influenzae* plays a role in susceptibility to severe infection It is not known whether the early development of severe infection is a reflection of inefficient antibody production the large size of the infecting dose or whether it represents depression of defense mechanisms by nonspecific forces such as stress cold nutritional depletion by starvation or by another infectious agent for example a virus In the 1957 Asian influenza epidemic we encountered not only a new clinical pattern of fulminating *H influenzae* infection of the central nervous system but also an increased incidence of severe infections During one week of January 1957 7 patients with *H influenzae* epiglottitis were admitted to Babies Hospital a few more than we usually see during 1 year

Virulence

Potential virulence is related to the encapsulated state of *H influenzae* and to the quantity of specific soluble substance elaborated During the 30 year period since Pittman s contributions on differentiation of disease producing strains and those which inhabit the nasopharynx of the majority of normal individuals the evidence has become even more convincing that *H influenzae* pyogenic disease is caused virtually always by type specific encapsulated strains The exceptions have been described

The distinctive ability of type *b H influenzae* to produce severe pyogenic disease with a high incidence of bacteremia may be due to the quality or quantity of its type specific substance a polyribophosphate or to other antigens or virulence factors

Mouse protection tests have been of some value for testing virulence but the need for suspension in mucin makes the infection unnatural and limits interpretation However virulence of nonencapsulated strains tested under these circumstances is lower than virulence of encapsulated type specific *H influenzae* On the other hand it has not been possible to show by this technic that type *b* has a higher virulence for the mouse than do the other specific types *a c d e* and *f*

Ecology

Many studies have demonstrated the presence of nontypable *H influenzae* in the nasopharynx of the majority of normal individuals in most age groups The relationship of typable potentially pathogenic hemophilus to the widely disseminated nontypable strains has assumed great interest and importance since the demonstration of in vitro DNA mediated genetic transfer from one organism to another Genetic traits of type *b H influenzae* indistinguishable from naturally occurring strains can be induced in vitro in nontypable *H influenzae* or in *H influenzae* organisms of different type specificity as a result of exposure to DNA isolated from type *b H influenzae*

When cultures of encapsulated strains of *H influenzae* are sparsely seeded on a transparent medium (Levinthal or Fildes agar) 2 kinds of colonies appear as first reported by Pittman (1931) Almost all of the colonies are opaque and large after 18 hours growth When viewed in obliquely transmitted light they show a characteristic iridescence At times there are present at least one or more colonies which fail to show iridescence they are smaller bluish and transparent Transfer of one of the latter colonies to Levinthal broth yields a culture with characteristics differing from the original no specific soluble substance is detectable by the usual criteria capsular swelling cannot be demonstrated and when the broth inoculum is again seeded on Levinthal agar no iridescent colonies are seen These variants arise spontaneously under what are deemed to be optimal conditions of artificial cultivation Under certain less favorable con

ditions the culture is made up predominantly of these variants and the culture is said to have passed from the smooth to the rough phase

The emergence of R cells from type *b H influenzae* has also been demonstrated in the nasopharynx of patients following recovery from influenzal meningitis Evidence that the R cells can be derived from type *b* organisms has in some cases been provided by a genetic marker in the R cells the inherited streptomycin resistant trait which the type *b H influenzae* cells grown from the spinal fluid and the nasopharynx also exhibited following treatment of a patient with meningitis by streptomycin alone

There is reason to believe that most children recover from type *b H influenzae* respiratory infections without specific therapy and develop immunity as a result The most rewarding group for study of the ecology of type *b H influenzae* are members of families in which one child has developed meningitis (Good *et al* 1943) all of the siblings will show type *b H influenzae* in the nasopharyngeal mucus some with respiratory tract signs and symptoms The mother usually shows this organism too from time to time whereas in the children it is persistent over long periods whenever they are cultured yet serious disease in more than one member is unusual Type *b H influenzae* may spread among patients on a hospital ward not unlike pneumococci (Johnson and Fousek 1953)

Transformation

There are at least 2 kinds of phenomena which are exerting forces responsible for changes in heritable traits of hemophilus as well as other populations (1) selection of spontaneously occurring mutants which is so well exemplified by emergence of resistance of *H influenzae* to streptomycin (Alexander and Leidy 1947a b) and (2) induction of new traits by exposure to a DNA derived from cells possessing the character to be introduced When nontypable *H influenzae* populations derived from types *a b d* or *e* but lacking capsules and ability to produce specific soluble substance and iridescent growth on Levinthal agar are exposed to DNA-containing extracts of type *a b c d e* or *f* certain genetic traits of the donors of the DNA will appear in the recipient populations Type-specific populations can be changed to a new type by exposure to DNA from a heterologous type Therefore absence of type specificity is not a prerequisite for induction of a genetic change (Alexander and Leidy, 1951b) In populations of either typable or nontypable *H influenzae* which are sensitive to streptomycin (Leidy Hahn and Alexander 1953), resistance of a high degree (1,000 mcg per ml) can be induced by DNA derived from populations which show the latter degree of resistance to streptomycin When type *b H influenzae* was exposed to DNA extract of type *a* cells an interesting phenomenon occurred type *a* and type *b* antigens could be demonstrated within the same cell designated Sab (Alexander and Leidy, 1953b) The DNA extract of Sab cells could induce the ab trait in R cells derived from type *d* and individual type *a* and type *b* traits in other cells These data have been interpreted as evidence of linkage of the heredity determinants of types *a* and *b* Thus the DNA s of *H influenzae* have been shown to function as heredity determinants in vitro

Whether this phenomenon occurs in vivo is not known at present If it can be shown to operate in vivo, the large reservoir of nontypable *H influenzae* found in the respiratory tracts of individuals of all ages may prove to be of greater significance than is now appreciated

In the hemophilus system in populations known to contain susceptible cells the reaction between the recipient cell and DNA is virtually immediate and does not require growth However even under the best conditions according to our present knowledge only a very small proportion of the population exposed is susceptible to induction of a genetic change The factors which influence the susceptibility or the competence of cells have assumed great significance

The evidence suggests that in a given population the size of the proportion of cells susceptible to different heredity determinants is the same (Alexander and Leidy 1954) When a population is exposed to mixtures of two different DNA s 75 per cent of the type *b* DNA and 25 per cent of DNA of type *c* 75 per cent of the transformed cells will be

type *b* and 25 per cent type *c* Moreover the action of one DNA previously can completely exclude a DNA added within 15 minutes

A number of factors have been shown to influence the proportion of a population transformed (Alexander *et al* 1954)

Type of Origin of Recipient Cells Type *a* exhibits the lowest frequency about 1 10 000 000 and type *d* the highest approximately 1 to 5 per 100 cells exposed This type specific property which controls the frequency of susceptible cells is an inherited trait repeated change to a heterologous type shows no influence on their incidence

Concentration of DNA Within certain limits increase in the concentration of DNA controlling streptomycin resistance can increase the size of the proportion of cells in which streptomycin resistance can be induced This result is to be expected if a single unit or molecule is the cause of transformation However 100 fold increases in concentrations greater than 10^{-1} μg per ml have not induced streptomycin resistance in a higher proportion of cells

Phase of Growth Cycle Predictable fluctuations in frequency of induced heritable changes have been demonstrated in R*a* R*b* R*d* and R*e* populations during growth in stationary Levinthal broth These fluctuations reflect physiologic changes in the cells Susceptible cells emerge in all experiments when the population reaches the end of the logarithmic period and a density of 2 to 4×10^8 cells per ml In the early logarithmic phase it is difficult to demonstrate the presence of any susceptible cells The peak frequency of cells susceptible to transformation to streptomycin resistance 0 1 per cent occurs in the early stationary phase of the growth cycle Thereafter the decline in frequency is gradual

Goodgal and Herriott (1961) have shown that the proportion of competent (transformable to streptomycin resistance) cells in R*d* *H influenzae* populations may be increased to 1 per cent or more if the population is aerated during exponential growth and then after a period of incubation with reduced aeration is diluted 1 20 into a medium containing DNA and then incubated for 30 minutes Some unknown factor(s) in the culture medium appeared to be essential for the high degree of competence of the diluted population in our experience dilution of even 1 100 in saline leads to a marked decrease in the transformability of the population This dilution effect was used as a tool for the development of a chemically defined medium in which a high degree of competence emerges even in dilution by a factor of 10^4 in 3 species of hemophilus *H influenzae* *H aegyptius* and *H parainfluenzae* The results suggest that 2 amino acids L aspartic and L glutamic play a significant role The process required for emergence of competence in the defined medium is temperature dependent and is inhibited by chloramphenicol (Leidy *et al* 1962)

Genetic Relationship of Recipient and Donor Cells DNA mediated resistance to streptomycin is induced in the highest proportion of a sensitive population of hemophilus when the donor of the DNA belongs to the same species as the recipient cells The ratio

$$\frac{\text{cells transformed by heterologous DNA}}{\text{cells transformed by homologous DNA}}$$

is a reflection of the degree of kinship or homology of genes of the recipient and the donor cells Our own work and that of others has suggested that the degree of reactivity of the DNA s of recipient and donor cells might be used as a taxonomic guide (Leidy *et al* 1956 Schaeffer 1958 Marmur 1963) This principle has been applied to a species of hemophilus previously known as Koch Weeks bacillus but reclassified as a new species *H aegyptius* the size of the ratio of transformants induced in an *H influenzae* population by DNA from *H aegyptius* suggests that *H aegyptius* is a variant of *H influenzae* (Leidy *et al* 1959) However differences between *H influenzae* and *H aegyptius* are well recognized Recent experiments in our laboratory designed to explore these differences have used 2 genetic markers resistance to streptomycin and resistance to novobiocin The greater sensitivity of *H aegyptius* to novobiocin is associated with a lower degree of resistance of the organisms emerging as a result of a single mutational step On the other hand the degree of genetic homology is clearly

demonstrated by the use of the 2 markers streptomycin and novobiocin resistance which are linked in each species (Leidy *et al* 1964)

Unknown Factors Recent reports indicated that the environment in which the population reaches the end of the logarithmic phase plays a role (Goodgal and Herriott 1961) For example, if rapid growth is promoted by increased oxygenation followed by decreased oxygenation and DNA exposure occurs in the presence of 0 15 M NaCl in 1 10 dilution the highest proportion of cells is transformed As the dilution is increased in saline this proportion decreases This decrease on dilution in saline can be prevented by the presence of 2 amino acids (Leidy *et al* 1962) The results suggest that certain metabolic reactions are necessary for completion of the transformation started in the salt environment

TREATMENT

Prior to 1938 the mortality in patients with influenzal meningitis was close to 100 per cent Today there are a number of effective therapeutic agents type specific rabbit antiserum (Alexander *et al* 1942) streptomycin erythromycin ampicillin the tetracyclines chloramphenicol and polymyxin The location the duration and the severity of infection govern the selection of agents in a given patient In febrile upper respiratory tract infection with early otitis media or sinusitis in obstructive epiglottitis and in pneumonia prompt recovery has occurred following use of sulfonamides alone However the risk of therapeutic failure is virtually eliminated by the use of an additional agent tetracyclines or chloramphenicol In the more serious infections meningitis pyarthrosis with or without osteomyelitis empyema or pericarditis the use of two agents is clearly indicated

Sufficient data are now available for a comparison of 3 different therapeutic programs in meningitis (1) type specific rabbit antiserum and sulfonamides (2) streptomycin and sulfadiazine and (3) chloramphenicol and sulfadiazine (Alexander 1956) Any of the 3 can be expected to cure virtually 100 per cent of the patients who are treated early in the course of the disease

Type Specific Rabbit Antibody Therapy This form of therapy is of interest today primarily because of its historic aspects and because it exemplifies a number of important principles in microbiology

Type *b H influenzae* rabbit antibody was the first therapeutic agent which made it possible to cure most patients suffering from type *b H influenzae* meningitis Horse antibody made in two other institutions had failed Whether the greater efficacy of rabbit antibody is explained by the qualitative differences between antibody produced in the rabbit and that made in the horse or whether our regimen for use of rabbit antibody satisfied the quantitative needs more effectively is not known The rabbit antibody which proved to be successful was measured quantitatively in terms of mg of agglutinin nitrogen per ml the size of the therapeutic dose in children differed with the severity of the infection the total amount judged to be necessary was administered at one time and after administration the adequacy of the dose used for a given patient was checked by examining the serum for its capacity to cause capsular swelling of young cultures of type *b H influenzae* (Alexander *et al* 1942) Horse antiserum had not been used according to these principles Moreover there is reason to believe that the chemical nature of the antigen used to produce rabbit antibody was more representative of the organism causing the infection and that therefore the rabbit antibody was a more specific therapeutic agent

Today other treatments are at least as effective for most patients with influenzal meningitis they are simpler to use are less expensive and do not have the disadvantage of causing serum sickness There is only a small proportion of patients with influenzal meningitis in whom there is a rationale for using specific rabbit antibody in addition to other agents mainly those who run a fulminating course and exhibit signs of widespread encephalitis during the first several hours after onset and young infants who exhibit signs of severe disease

Streptomycin Streptomycin as a single therapeutic agent is seriously limited in its capacity to eliminate *H influenzae* in central nervous system infection when the patient exhibits signs of severe infection—in other words when the bacterial population is suf-

ficiently large Under this circumstance mutants resistant to high concentrations of streptomycin emerge rapidly This deficiency can be eliminated by using a sulfonamide in conjunction with streptomycin

Two other disadvantages of streptomycin deserve consideration in our experience (1) Optimal concentrations of streptomycin can be attained at the site of infection only by intrathecal administration of streptomycin Today intrathecal treatment can be eliminated in most patients with meningitis by choice of an appropriate therapeutic regimen (2) Damage to the vestibular branch of the 8th nerve and to the auditory branch are hazards of streptomycin treatment which no longer need to be accepted

Chloramphenicol and Sulfonamides There is general agreement that chloramphenicol in conjunction with a sulfonamide is now the program of choice Both agents are found in the spinal fluids in optimal concentrations promptly following parenteral administration the levels are approximately two thirds of the concentrations found in the blood Therefore intrathecal treatment is unnecessary The concentration of chloramphenicol found in the spinal fluid is bactericidal In the more than 10 years during which chloramphenicol and a sulfonamide have been used for treating patients with influenzal meningitis the spinal fluid after a 24 hour treatment has been sterile and we have encountered no recrudescences Our program is as follows chloramphenicol in a dose of 100 mg /K daily (not to exceed 2 G) and sulfisoxazole in a dose sufficient to maintain a concentration of 10 mg per cent are given intravenously for the first 24 hours along with 5 per cent dextrose and enough physiologic saline and water to satisfy requirements of the individual

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30

The Bordetella Group

BORDETELLA PERTUSSIS

Bordetella is a newly created genus which includes *B pertussis B parapertussis* and *B bronchiseptica* all formerly included in the genus *Hemophilus* The separate genus was proposed because these organisms do not always require the growth factors found in blood and because they are antigenically related

B pertussis is the cause of whooping cough a highly communicable acute infection of the respiratory tract It is prevalent among infants and children and may cause serious pulmonary and cerebral complications and even death especially in young infants

History

Little is known about whooping cough until the Middle Ages when Moulton described drugs used in the treatment of the kink a Scottish colloquialism synonymous with fit or paroxysm It was also known as chin-cough derived from the teutonic word *kindhoest* meaning child s cough DeBaillau 1578 is credited with the first classic description of the disease (an epidemic in Paris which was called *coqueluche*)

One of the earliest American descriptions was that by Benjamin Waterhouse 1822 A comprehensive account of the disease may be found in Lapin s Whooping Cough 1943

The causative organism *B pertussis* was first observed and isolated from an infant by Bordet and Gengou (1906) Chievitz and Meyer (1916) introduced the cough plate method for bacteriologic diagnosis A more practicable and efficient method now in general use is the nasopharyngeal swab technic (Bradford, Day and Berry 1946)

The early ineffectiveness of pertussis vaccine was explained by Leslie and Gardner (1931) whose work stressed the changes in antigenic phases that occur during artificial cultivation of the organism

Although the dual etiologic role of a virus was suggested by certain investigators it is now clear that *B pertussis* alone can produce the disease in laboratory animals (Shibley 1934) as well as in man (MacDonald and MacDonald 1933)

Morphology

B pertussis, when first isolated is a small nonmotile ovoid bacillus Its mean length like that of *B parapertussis* or *B bronchiseptica* is 0 5 micron Cells of original cultures are uniform in size but on subculture become pleomorphic longer and thread shaped In liquid media a ropy mucoid mass results from the growth of old cultures Electron micrographs of the organism reveal a central mass of dense material surrounded by a wide clearer zone which is said to contain antigen

The organism is gram negative staining best when the counterstain is left on for 2 minutes Capsules may be demonstrated by special technic and bipolar metachromatic granules said to represent uneven distribution of cell lipids can be shown by staining

with toluidine blue as suggested by *Bordet* (Sauer 1957)

Cultivation and Biochemical Reactions

Primary isolation is obtained best on complex media such as that originally used by Bordet and Gengou consisting of a potato blood agar glycerol mixture or some modification of it It should contain at least 15 per cent blood Charcoal may be used to replace blood in certain agar media (Pollock 1947) because it like albumin neutralizes toxic fatty acids which inhibit growth The optimal temperature for growth is from 35 to 37° C

A solid medium which has proved to be satisfactory is prepared as follows

1 Bordet Gengou Agar Base dehydrated *Bacto (Prepared by the Difco Laboratories Inc Detroit)* This base contains the following ingredients per liter

Potato infusion from	125 Gm
NaCl	5 5 Gm
Proteose peptone Difco	10 0 Gm
Bacto-agar	20 0 Gm

2 Solution of 1 per cent glycerol in distilled water

3 Freshly withdrawn (not over 6 hours) defibrinated sheep s blood

A Suspend 4 Gm of the dehydrated agar base in 100 ml of 1 per cent solution of glycerol in distilled water Heat to boiling to dissolve the medium completely Sterilize in autoclave at 15 lbs pressure (121 C) for 20 minutes This base may be stored in 100 ml Erlenmeyer flasks in the refrigerator

B To prepare the final medium heat the base prepared as described above in a water bath until completely liquefied Cool to from 45 to 50 C by placing the flask in a water bath and add the blood to make a concentration of from 20 to 25 per cent and pour plates Use plates for cultures which have been prepared within 72 hours

Satisfactory fluid media have been described by Cohen and Wheeler (1946) by Verwey (1949) and by Sutherland and Wilkerson (1961)

B pertussis produces no gas and does not attack carbohydrates It neither forms indol nor reduces nitrates In litmus milk alkalinity results in 10 to 14 days as compared with 1 to 4 days in the case of *B parapertussis* or *B bronchiseptica* About 70 per cent of the strains are catalase positive compared with nearly all strains of *B parapertussis* Citrate is not utilized and urea is not split whereas *B parapertussis* can utilize citrate as the sole source of carbon and readily splits urea

Antigenic Relationship

Bordet and Sleeswyk 1910 observed that all recently isolated strains of *B pertussis* agglutinated in a serum prepared against any one of them thus suggesting a single antigenic type Subsequent workers have generally confirmed this observation However when such strains of the organism are maintained on nutrient agar variant forms occur Leslie and Gardner (1931) *from a study of* 32 apparently smooth strains described 4 *different antigenic phases I the virulent S* form IV the completely avirulent form and *II and III intermediate serologic variants* Of 20 recently isolated strains 18 were *found to be in* Phase I

Some investigators regarded these variations in antigenic relationship as simply changes in the S → R forms in which varying amounts of Phase I antigens remained on the surface of the bacteria others believed that qualitative differences existed with the various phases as did Leslie and Gardner

The nature of the change in the surface antigens of Phase I organisms during subculture is not clear A rearrangement in the proportion of the different antigens a modification of these antigens or the appearance of new ones have all been suggested

Several antigenic components of the S form are now recognized agglutinogen toxins (heat labile and heat stable) hemagglutinins and protective antigen

The agglutinogen (Flosdorf *et al* 1940 Smolens and Mudd 1943) is water soluble and can be liberated from the cell by sonic vibration and by acid extraction Injected into rabbits it elicits a high titer of agglutinin it removes this antibody from such serum by absorption It is nontoxic

Andersen (1953) by observing the resistance of various strains to heating at 100 C described 2 antigens one thermostabile (O) the other thermolabile (K) He found that all strains of *B pertussis* had a

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vades by continuity the bloodstream and even the middle ear are seldom involved However the latter often becomes infected with other organisms

Experimental infection can be produced in a number of species including the chimpanzee monkey puppy rabbit rat mouse ferret and chick embryo

From experimental infection it is clear that when Phase I and II *B pertussis* is introduced intra abdominally (rabbit or guinea pig) the results are more toxic than invasive (Leslie and Gardner 1931) More avirulent forms (Phase III or IV) are from 20 to 30 times less toxic

The intranasal instillation into mice gives rise to a characteristic patchy interstitial pneumonia with leukocyte infiltration, proliferation of bronchial epithelium and other changes not unlike those seen in the human disease (Burnett and Timmins 1937 Bradford 1938) This type of experimental infection has made available a useful method for immunologic study (Cooper 1952 Bradford 1956)

The intracerebral route of infection was introduced by Kendrick *et al* (1947) as a means of testing degrees of immunization in mice Comparative virulence tests according to the route of infection in the mouse were reported by Standfast (1951) as follows

Route of Infection	Range of I D50 in Millions of Bacteria
Intraperitoneal	280 800
Intranasal	0 26 15 5
Intracerebral	0 18 2 5

It is perhaps significant that Andersen (1952) found a nontoxic variant after intra cerebral inoculation which was highly virulent for mice producing septicemia

Study of toxic preparations may be carried out by intradermal injection into rabbits and 10 day old mice (Katsampes *et al* 1942 Andersen 1953)

In man the infection is variable in its course but 3 stages may be observed the catarrhal the paroxysmal and the convalescent each lasting approximately 2 weeks The catarrhal stage begins with coryza sneezing and a mild progressive cough about 10 to 14 days after exposure The organism multiplies rapidly on the mucus membranes of the respiratory tract and soon produces necrosis of the basilar and the midzonal portions of the bronchial epithelium with infiltration of leukocytes As the infection extends to the deeper structures peribronchiolitis and interstitial pneumonia results Intra alveolar and localized suppurative lesions are usually caused by secondary invading organisms Edema and hemorrhage often occur in the lung parenchyma

Obstruction of lower airways by mucous plugs causes atelectasis which along with interstitial pneumonia interferes with oxygenation of the blood and leads to acidosis The resulting anoxia is said to be an important cause of convulsions The exact mechanism responsible for postpertussis encephalitis is not known although there is experimental evidence to support an allergic factor

Experimental encephalomyelitis has been produced by substituting *B pertussis* for *M tuberculosis* in the Freund antigen (Weiner Tinker and Bradford 1959)

The heat labile toxin may be responsible for lymphocytosis early necrosis of the bronchial epithelium and certain hemorrhagic manifestations

The possible role of allergy in the pathogenesis of the disease has been suggested by Toomey (1938) Parfentjev (1947) showed that mice and guinea pigs injected with *B pertussis* antigens developed sensitivity to the nucleoprotein complex of the organism Pittman (1951) found that mice infected intranasally with the organism exhibited a high degree of sensitivity to histamine Ehrlich *et al* (1942) showed that injection of a sonic extract of the organism into rabbits resulted in leukocytosis which was almost exclusively a lymphocytosis However when mice recovering from the intranasal infection were injected with a whole sonic extract or with such an extract from which agglutinogen had been removed by absorption leukocytosis resulted due chiefly to a polymorphonuclear increase (Bradford Scherp and Tinker 1956)

Immunity

Permanent immunity appears to follow the natural disease Bacteriologically proved instances of second attacks have been noted but are rare Little or no maternal immunity

TABLE 1 TENTATIVE ANALYSIS OF ANTIGENIC FACTORS OF BORDETELLA CULTURES*

CULTURE	FACTORS
B pertussis	
5373	7 1 3 6 13
5374	7 1 2 5 6 13
5375	7 1 2 4 13
B parapertussis	
17 903	7 8 9 10 14
B bronchiseptica	
5376	7 8 9 12 13
214	7 9 12 13
899	7 8 10 11 12

* From Eldering *et al* (1962) J Bact *83* 745 9
All *B pertussis* cultures have factor 1 Factor 12 is species specific for *B bronchiseptica* and factor 14 for *B parapertussis* Factors 2 3 and 5 are major *B pertussis* antigens and serologic differences among *B pertussis* cultures may be explained by the presence or the absence in various combinations of these factors Factors 4 and 6 are minor *B pertussis* antigens Factors 8 9 10 and 11 distinguish different *B bronchiseptica* cultures and all of these except 11 are also possessed by all *B parapertussis* cultures A minor factor 13 explains certain serologic relationships between *B pertussis* and *B bronchiseptica* This antigen is lacking in *B parapertussis*

common O antigen but differed in their content and number of K antigen Furthermore Andersen concluded that *B pertussis B parapertussis* and *B bronchisepticus* have common O antigens and produce similar hemorrhagic toxins but may possess common as well as species specific K antigens *B pertussis* apparently possesses more antigens in common with *B bronchisepticus* than it does with *B parapertussis*

Eldering Eveland and Kendrick (1957 1962) have confirmed and expanded Andersen s findings and propose hypothetical factors concerning the antigenic structure of the *Bordetella* group (Table 1)

The toxin (Evans and Maitland 1937) when injected intradermally into rabbits produces necrosis and is lethal for guinea pigs It is not stable and is destroyed by heat ing at 55° C When formalinized it is antigenic and its antitoxin neutralizes the toxins of *B pertussis B parapertussis* and *B bronchiseptica* While produced by all phases of *B pertussis* toxin is most abundant in Phase I organisms and only one tenth as abundant in avirulent strains (Flosdorf and McGuinness 1942) Disruption of the organism gives the best yield of toxin but an appreciable amount is contained in the surface washings of the freshly isolated organisms (Katsampes *et al* 1942) Purified heat labile toxin is chiefly protein (Banerjea and Munoz 1962)

The heat stable toxin probably of intracellular origin is only partially destroyed by heating at 100° C for 60 minutes Little is known of its nature and properties

Hemagglutinin (Keogh North and Warburton 1947) can be extracted from the freshly isolated organism Antisera to these extracts specifically neutralize their hemagglutinin effect Keogh found that resistance to infection parallels antihemagglutinin production but Masry (1952) reported that purified hemagglutinin was not protective and Standfast (1951) found no correlation between virulence and hemagglutinin

Protective antigens have been described and are the subject of controversial opinion Cruickshank and Freeman (1937) and Eldering (1942) isolated carbohydrate antigens that protected mice while Flosdorf and Kimball (1940) and Smolens and Mudd (1943) considered the protective effect to be associated with the agglutinogen Capsular material of *B pertussis* has no significant protective value (Evans and Adams 1942) Robbins and Pillemer (1950) isolated a purified protective antigen from a watery extract of the bacillus which was not carbohydrate agglutinogen toxin or hemagglutinin It appears to be generally agreed that the protective antigen is associated with the smooth forms of the organism and is more abundant in young rather than old cultures

HOST RANGE AND PATHOGENESIS

B pertussis is an obligate parasite which survives only a short time outside the human host It survives only a few hours in dried sputum and is killed in 30 minutes by a temperature of from 50° to 55° C It is very sensitive to ultraviolet light and to chemical antiseptics

Transmission is by droplet infection and the human carrier plays a minor role While the organism multiplies rapidly in the mucus membranes of the respiratory tract and in

colonies well formed in 48 hours with a darker area of discoloration about them

The application of fluorescent antibody technic (Donaldson and Whitaker 1960 Kendrick Eldering and Eveland 1961) offers promise but requires further refinement.

Agglutinins (often 1 160 or higher) appear during the 3rd week of infection and may persist for several months Because they appear so late they are of little practical value in diagnosis

Skin testing with purified agglutinogen often results in a positive reaction in the immune which is said to correlate with complement fixing antibody titer and with the agglutinative titer So far this skin test has not received wide acceptance

Clinically a history of exposure a progressive cough that becomes paroxysmal and often ends in vomiting or a forced inspiration (the whoop) are highly indicative of pertussis The total white blood cell count usually increases during the end of the catarrhal stage The leukocytes may number 15 000 to 30 000 per ml reflecting a relative and absolute predominance of lymphocytes

Other conditions which resemble pertussis are spasmodic coughs resulting from infected adenoids or sinuses allergic bronchitis bronchopneumonia atypical viral pneumonia mucoviscidosis and mediastinal lesions Parapertussis resembles mild pertussis and can be differentiated only by culture Infections caused by *B bronchisepticus H influen ae* and *Brucella abortus* have been mistaken for pertussis

Treatment

Mild and moderately severe pertussis requires only supportive measures but severe cases require the best of professional care usually in a hospital Hyperimmune human serum (McGuinness 1944) injected intramuscularly in doses of from 20 to 40 ml or the gamma globulin produced from it result in humoral antibody levels which compare favorably with those observed in convalescence from the natural disease An injection of vaccine during the incubation period or the early catarrhal stage of the disease may have a beneficial anemnestic response

In vitro various drugs inhibit growth of *B pertussis* according to Wells *et al* (1950) In micrograms per ml the range of concentrations is streptomycin 0 8 to 80 polymyxin 0 08 to 1 0 chlortetracycline 0 16 to 12 5 chloramphenicol 0 16 to 80 and oxytetracycline 0 2 to 12 5

From clinical trial Booher *et al* (1951) concluded that chloramphenicol and the tetracyclines are of about equal value when given in dosages of 50 mg per Kg of body weight

In another clinical trial Ames *et al* (1953) tested streptomycin chloramphenicol antipertussis rabbit serum and hyperimmune human serum Each agent showed some modifying effect but none was of convincing therapeutic value Chloramphenicol appeared to be the agent of choice Rabbit antiserum gave the most rapid clearance of the organism from the nasopharynx Penicillin is effective only against susceptible secondary invaders

Epidemiology

Pertussis exists sporadically and endemically throughout most of the world In the United States epidemics tend to occur at intervals of from 2 to 4 years Although it occurs at all seasons most cases are observed during the early winter months in the Northern States and during the spring months in the South The communicability rate in family exposure is about 90 per cent in schoolroom exposure about 25 to 50 per cent This is high for a bacterial disease and is approximately that of such viral diseases as measles and chickenpox

From 1920 to 1959 the mortality from pertussis in the United States decreased from 12 5 to 0 2 per 100 000 population and has been below 1 during the past 12 years The death rate is higher in rural than in urban areas and is highest in early infancy

The disease occurs at all ages About one half of those infected are under 4 years of age about 10 per cent are under 1 year Sixty four per cent of the deaths are under 1 year of age while 40 per cent occur during the first 5 months of life

Pertussis is unique in that its incidence is significantly greater among females especially in the later years of childhood Mortality also is higher among females

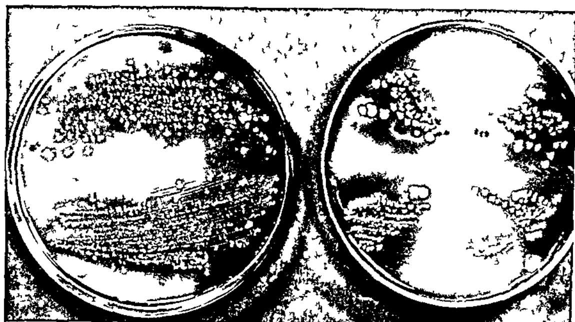


FIG 1 Nasal culture method showing the inhibiting effect of penicillin on the growth of contaminating organisms (*right*) to facilitate the identification of *Hemophilus pertussis* colonies on the surface of Bordet s medium The control plate is shown on the *left* (Bradford W L Day E and Berry G P 1946 Improvement of the nasopharyngeal swab method of diagnosis in pertussis by use of penicillin Am J Pub Health *36* 468)

is passively transferred to the newborn Indeed early infancy is a period of great susceptibility and of highest mortality

The relative importance of humoral and cellular immunity is not known Humoral antibodies, as measured by complement fixation agglutination opsono—cytophagic and mouse protective tests appear during convalescence from an attack and as a result of immunization and persist for several months Sako (1947) observed immunized infants subsequently subjected to family exposures and compared the attack rate with their humoral agglutinative titers The results indicated a positive correlation as follows

Serum Titers	Number of Cases	Percentage Attacked
1 320	149	0
1 160	53	11 3
1 40	91	18 7
0	27	33 3

Among the nonimmunized controls with O titers the attack rate was 89 7 per cent

When tested intradermally with purified agglutinogen the reaction is usually negative in susceptibles and positive in immunes There appears to be a certain agreement between positive skin tests to this agent and significant humoral agglutination titers

DIAGNOSIS

Bacteriologic diagnosis can be made by the cough plate culture or by the nasal swab method (Fig 1) The cough plate is exposed by holding it about 6 inches from the mouth of the patient while he coughs The nasal swab is passed through the nasal aperture until it touches the posterior pharyngeal wall Upon withdrawal it is passed through a drop of penicillin previously placed on the surface of Bordet medium and the inoculation is streaked with a flexible platinum loop After incubation for 2 to 3 days at 36° C the characteristic colonies of *B pertussis* are readily apparent especially in the area where the contaminants are inhibited by the penicillin The organism is identified by staining and by agglutinative reactions with specific antiserum

B parapertussis by this technic gives larger

plain agar and in liquid media suitable for the latter organism

Practically all strains produce catalase (Lautrop 1954) It does not produce indol or hydrogen sulfide nor does it reduce nitrates Milk is made alkaline more readily and to a greater degree than by *B pertussis* All strains examined by Lautrop (1954) split urea while none of more than 500 strains of *B pertussis* did

B parapertussis possesses common antigenic fractions with both *B pertussis* and *B bronchiseptica* but is identical with neither (Fig 2) Cross agglutination between *B parapertussis* and *B pertussis* is caused by a common minor antigen (Flosdorf 1942) Each of these 3 species has its own thermostable surface antigens but all have a common thermostable O antigen (Andersen 1953) *B parapertussis* produces a toxin similar to but less potent than that of *B Pertussis* (Bruckner and Evans 1937)

Experimental infection is easily produced in mice by intranasal inoculation and is characterized by moderate leukocytosis and pulmonary lesions resembling those produced by *B pertussis* (Bradford and Wold 1939)

The incubation period in man is from 6 to 15 days The onset is similar to that in whooping cough but may be more abrupt The cough is less severe frequently spasmodic and sometimes it resembles that in tracheitis The duration of illness is usually from 1 to 3 weeks and complications are rare although fatal pneumonia has been reported (Zuelzer and Wheeler 1946)

The mildness or the absence of symptoms in certain subjects harboring the organism led Lautrop (1954) to suspect the frequent existence of carriers

Reciprocal immunity with pertussis does not exist and there is no evidence that immunization with pertussis vaccine protects against parapertussis It is not known that an attack of the disease confers lasting immunity although second attacks have not been reported

Treatment is symptomatic The organism is susceptible although to a less degree than *B pertussis* to chloramphenicol and the tetracyclines (Day and Bradford 1952) and one of these agents should be used for therapeutic purposes Because of the usual mildness of the disease active immunization has not as yet been regarded as necess ry

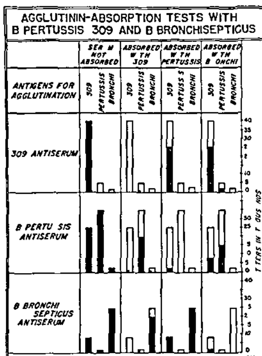


FIG 2 The antigenic relationship of *H parapertussis* (309) with *H pertussis* and with *Brucella bronchiseptica* (Eldering G and Kendrick P 1938 *Bacillus parapertussis* A species resembling both *Bacillus pertussis* and *Bacillus bronchiseptica* but identical with neither J Bact 35 561 572)

BORDETELLA BRONCHISEPTICA

Bordetella bronchiseptica was first isolated from dogs with distemper (Ferry 1911 McGowan 1911) in which it is now regarded as a secondary invader It causes infection of laboratory animals (guinea pigs rabbits etc but not mice) In man particularly those individuals in contact with infected animals it occasionally causes a respiratory infection resembling pertussis (Brown 1926 Kristensen and Lautrop 1962)

B bronchiseptica is a small (1 to 3μ) motile gram negative and non spore producing bacillus It does not require X and V

CONTROL MEASURES

Effective control of whooping cough requires early diagnosis proper management of the case and contacts and an adequate program for active immunization Because of the insidious onset and variable course diagnosis is delayed longer than in most other common communicable diseases Early recognition is important because the patient is most infective in the initial stage of the disease Culture facilities should be more widely available and used for this purpose

The patient should be isolated for a period of from 4 to 6 weeks and ideally should show a negative culture before release Susceptible exposed subjects should be isolated for 2 weeks These especially infants should receive passive protection (gamma globulin produced from hyperimmune human serum) injected intramuscularly in doses of 2 to 5 ml The previously vaccinated exposed subjects should be given an additional injection of vaccine which gives an anamnestic response in from 4 to 6 days

An adequate program of active immunization should include all infants The initial course of vaccine is usually started at 3 months of age (or earlier) and consists of 3 or 4 monthly injections of vaccine totaling 12 antigenic units This vaccine is usually combined with diphtheria and tetanus toxoids and apparently is effective in a multiple antigen to which poliomyelitis vaccine is added Booster injections (2 to 3 units each) should be given 1 3 and 5 years after the initial course

Encephalopathy following injection of pertussis vaccine has been reported (Byers and Moll 1948) and some 60 instances have been observed This complication though serious should not prevent the routine immunization of infants Caution should be observed during immunization of one who gives a history of frequent convulsions particularly following a previous injection of vaccine Such individuals should receive the vaccine in small doses

BORDETELLA PARAPERTUSSIS

Bordetella parapertussis is a short ovoid gram negative, nonmobile bacillus which in many respects resembles *B pertussis* It is the cause of parapertussis an acute respiratory infection resembling mild pertussis, from which it can be distinguished only by bacteriologic methods

The organism was first described independently by Elderling and Kendrick (1937) and by Bradford and Slavin (1937) In each instance it was isolated from cases of suspected whooping cough A few strains, later recognized as *B parapertussis* were observed in Denmark in 1933 according to Miller The name *Bacillus parapertussis* was suggested by Kendrick (1938) who objected to its inclusion in the hemophilic group

B parapertussis was encountered originally (Bradford and Slavin 1937) in 5 per cent of a consecutive series of positive cultures for *B pertussis* From more than 22 000 diagnostic cultures (1935 1950) Eldering and Kendrick reported (1952) that 19 8 per cent revealed *B pertussis* and 0 5 per cent were positive for *B parapertussis* In Copenhagen Lautrop (1954) found 5 per cent of all cultures taken (representing 16% of all positive ones) positive for *B parapertussis* He observed 256 cases between November 1950 and March 1952

The demonstration of specific humoral antibodies among children in California (Miller) and in Philadelphia (Flosdorf) indicates the infection to be rather common and easily overlooked In a random sample of routine hospital admissions (531 infants and children in Rochester) 7 1 per cent revealed agglutinins against *B parapertussis* in titers of 1 320 or greater while 34 4 per cent had similar titers against *B pertussis* (Scherp *et al* 1954)

In schools, day nurseries and camps local outbreaks have been observed sometimes occurring with pertussis In family exposures Lautrop (1954) observed the following incidence according to age 0 to 7 years 85 per cent 7 to 17 years 35 per cent adults 10 per cent

B parapertussis on primary isolation grows readily on Bordet medium the colonies appearing within 24 to 48 hours They are smooth round and glistening and are surrounded by zones of dark discoloration No report of its primary isolation in bloodless media is as yet available On subcultures it grows more readily than does *B pertussis* on

A new growth medium for virulent *Bordetella pertussis* J Path Bact *82* 431 438

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MacLennan A P 1960 Specific lipopolysaccharides of *Bordetella* Biochem J *74* 398 409

factors but does require niacin for growth The colonies are small, round and have a glistening smooth surface Some strains are hemolytic It ferments no sugars, reduces nitrates splits urea easily and produces catalase

B bronchiseptica possesses common antigens with *B pertussis* and *B parapertussis* (Table 30 1), and with *Brucella abortus* Its specific toxin is neutralized by the antitoxin of *B pertussis* The specific lipopolysaccharide (MacLennan 1960) is antigenic and resembles those of *B pertussis* and *B parapertussis* in composition

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31

Listeria and Erysipelothrix

Listeria and *erysipelothrix* are gram positive rod shaped nonsporulating aerobic bacteria of the family *Corynebacteriaceae* which are pathogenic for a wide variety of mammals and birds While the disease entities produced by the two organisms are not similar the bacterial species appear to be related taxonomically Some authorities place them in the same genus although there is disagreement on this point

LISTERIA MONOCYTOGENES

History

Listeria monocytogenes is associated with sporadic cases of meningitis and granulomatosis infantiseptica in man and with a number of clinical syndromes in mammals and birds The organism was first isolated by Murray Webb and Swann (1926) from an epizootic among stock rabbits and guinea pigs and described by them under the name *Bacterium monocytogenes* in view of the mononuclear leukocytosis observed in these animals Pirie isolated an identical organism in 1927 from an epizootic of wild rodents in South Africa and created the generic name *Listerella* Later this was shown to be a homonym and the name *Listeria* was proposed by Pirie (1940) It seems probable that the same organism was isolated from the spinal fluid of patients with meningitis much earlier by Atkinson in 1917 and Dick in 1920 In recent years there has been a renewal of interest in both human and animal infections due to *L monocytogenes*, which have a widespread geographic distribution There is a much greater awareness of the possibility of such infections on the part of physicians veterinarians and bacteriologists alike A recent monograph by Seeliger (1961) lists 600 references to listeria

Morphology and Cultivation

L monocytogenes is a gram positive facultatively anaerobic rod approximately 0 5 micron in width by 1 to 3 microns in length The organism occurs in both a smooth and a rough form Small rods with rounded ends showing palisade formation and short chains predominate in the smooth phase Metachromatic granules are never observed Young cultures in the rough phase consist almost entirely of filaments up to 60 microns in length while older cultures contain many pleomorphic forms Young broth cultures show a characteristic sluggish tumbling end over-end motility Cultures grown at 37° C contain both nonflagellated and monotrichous organisms while those grown at room temperature are peritrichous with up to 4 flagellae *L monocytogenes* was considered to be acapsular until the demonstration by Smith and Metzger (1962) by phase microscopy of a mucopolysaccharide capsule in broth cultures containing 10 per cent rabbit serum and 5 per cent glucose

The organism grows best in a neutral or slightly alkaline evironment It will not tolerate acidity below pH 5 6 but good

growth occurs in fluid media up to pH 9 6 *L monocytogenes* grows well on most simple media although growth is improved markedly by the addition of ascitic fluid or blood Wel shimer (1963) has shown that riboflavine biotin thiamine and thioctic acid are required for growth Colonies in the smooth phase on nutrient agar are up to 0 8 mm in diameter after 24 hours at 37° C and are almost transparent by transmitted light Those in the rough phase are slightly larger with a granular center Both smooth and rough phase colonies may be up to 2 mm in diam eter on horse rabbit or human blood agar and most strains show a narrow zone of beta hemolysis On tellurite medium colonies are black smooth circular and 0 25 to 0 5 mm in diameter after 48 hours No growth occurs on MacConkey s agar Good growth occurs in infusion broth in which the smooth phase produces an even turbidity while the rough form gives a threadlike granular growth which does not disperse readily The bio chemical reactions of *L monocytogenes* are rather variable Production of acid without gas in 24 hours from glucose levulose trehalose and salacin is most constant and reliable (Gray 1962) The catalase reaction is positive in contrast with the negative test always given by *E rhusiopathiae*

Resistance

Listeria is less susceptible to heat than many pathogens since it has been shown to survive 5 minutes at 80° C (Murray 1955) Carefully controlled pasteurization of milk containing more than 1 000 bacteria per ml does not effectively destroy listeria (Bearns and Girard 1958) Listeria will survive with out loss of virulence for 3 to 4 years at 4 C either on culture media (Murray 1955) or in brain suspensions (Gray *et al* 1948) It will remain viable and virulent after 8 weeks in 20 per cent NaCl at 40 C and will grow well in 10 per cent NaCl but sur vival is short in normal saline or distilled water Listeria added to food pellets straw and wood shavings will survive up to 26 weeks in the dry state and there is evidence that it persists for at least 2 weeks in animal pens and bedding straw (Gray *et al* 1956)

The organism resembles most pathogenic bacteria in its susceptibility to the chemical agents ordinarily used as disinfectants McBride and Girard (1960) found that a medium containing 1/100 000 furacin while not providing complete inhibition of other organisms was a useful initial enrichment procedure for some types of specimens

Antigenic Structure

Agglutination and agglutinin absorption technics have been used extensively to study the antigenic structure of *L monocytogenes* Paterson (1940) recognized 3 somatic anti gens and a complex of flagellar antigens on the basis of which he established 4 sero logic types These results were confirmed by Seeliger and Linzenmeier (1953) who also demonstrated 2 subtypes in Paterson type 4 Types 1 and 4 appear to be world wide in distribution whereas type 3 is found mainly in Eastern Germany and Denmark and type 2 mainly in Great Britain (Seeliger 1961) Biotypes are recognized within types 1 and 4 based on their ability or lack of ability to ferment melizitose Some antigenic com ponents of *L monocytogenes* are common with those of other pathogens particularly *Staphylococcus pyogenes* and *Streptococcus faecalis* (Seeliger and Sulzbacher 1956 Neter *et al* 1960) The recently discovered capsule is antigenic and may further com plicate the serologic picture Bacteriophages have been isolated from listeria from both human and animal sources by Sword and Pickett (1961) who found the phage types to be very closely related to serologic types

There is at present no clear evidence than an exotoxin plays a role in the patho genesis of listeric infection *L monocyto-genes* contains a nonantigenic lipid which enhances antibody formation in animals im munized with other bacteria (Stanley 1949) More recently protein preparations obtained from lysates (Patočka *et al* 1959) and sonically disrupted (Silverman *et al* 1961) listeria have been shown to reduce the re sistance of laboratory animals to listeria and to other organisms A soluble antigenic hemolysin has been described in some detail by Girard *et al* (1963) It has been noted by Fuzi and Pillis (1962) that listeria pro duces opacity in media containing native egg yolk and that the degree of opacity parallels the degree of hemolysis on blood agar The

exact relationship of these substances to one another and to the pathogenicity of *L monocytogenes* remains to be determined Sheep can be protected against large challenge doses of listeria by prior subcutaneous inoculation of live virulent culture (Osebald and Sawyer 1955) or of living attenuated listeria (Asahi, 1963a)

Distribution and Range of Pathogenicity

Spontaneous infection of a wide variety of animal species including man, has been reported from many parts of the world *L monocytogenes* has been isolated and identified from many kinds of mammals fowls fish, crustaceans and from 2 species of tick its presence has been established in stream water sewage and silage (Gray, 1962) The majority of infections occur in domestic or captive animals and there also exists an abundant reservoir among wild species some of which are predatory and many of which are migratory *L monocytogenes* has been reported from nearly 30 countries ranging from the arctic to the tropics In view of the increasing number of infections in man and in livestock and poultry listeriosis is receiving increasing attention

Pathogenesis and Symptomatology

L monocytogenes infections produce many different symptoms depending on the animal species and the portal of entry

In cattle, sheep and goats meningoencephalitis of the brain stem is common producing frequently a flaccid paralysis of various muscle groups The lesions may extend to the spinal cord with resulting unilateral ataxia The head and the neck are turned to one side and the animal tends to move in circles, hence the common name of circling disease Asahi (1963b) has demonstrated invasion of the trigeminal nerve as a route to the brain in encephalitis Septicemia and monocytosis are uncommon in ruminants A generalized infection occurs in rabbits and guinea pigs with focal necrosis particularly in the liver and the adrenal glands the disease is usually accompanied by a marked monocytosis The mononuclear response in rabbits is apparently due to a nonantigenic lipid which can be extracted from listeria (Stanley, 1949, Girard and Murray, 1954) The natural disease in rabbits is accompanied by marked edema and extensive serous exudates, while in guinea pigs there is usually massive myocardial necrosis In the dog and the fox, listeriosis produces a distemperlike disease In the lemming, it appears as a generalized infection not accompanied by any obvious lesions although it is fatal

Abortion due to listeria has been described in sheep, goats, swine, rabbits and cattle In many instances the fetus shows extensive necrosis similar to that seen in newborn human infants In some of these cases the organism can be isolated from the aborted fetus, the placental membranes, or the vaginal discharge of the female In the chicken listeriosis is a septicemic disease often accompanied by massive myocardial degeneration and necrosis Listeria produces keratoconjunctivitis following conjunctival inoculation in rabbits guinea pigs and hamsters Cutaneous lesions have been reported in veterinarians after handling bovine fetuses infected with listeria (Gray, 1962)

Human listeriosis was once thought to be a rare disease and reviews of a decade ago listed only some 40 cases There is no evidence that its actual incidence is greater today but the number of laboratory diagnosed cases is now over 1,500 of which 4/5 have been recorded in the past 5 years (Gray, 1962) It is probably much more common than is realized since diagnosis depends on isolation and identification of the organism which is not always easy The various types of human listeriosis fall approximately into the following categories 33 per cent meningitis or encephalitis 29 per cent granulomatosis infantiseptica 21 per cent septicemia 8 per cent mononucleosis 6 per cent conjunctivitis (Murray 1955) Listeria meningitis is the most prevalent form of the infection in North America and has a fatality rate up to 70 per cent Clinically it resembles other purulent meningitides and the cellular response is mononuclear or polymorphonuclear in nature Although listeria is usually a primary cause of meningitis in adults it may sometimes be

superimposed on advanced neoplastic diseases diabetes or other terminal illnesses or may follow the administration of cortisone Septicemic cases occur under a variety of circumstances Listeriosis of the central nervous system is believed not to leave sequelae but Josephson (1963) has reported a well documented case in a 5 week-old infant which left residual spastic weakness and foot deformity The organism was once thought to play a role in the etiology of infectious mononucleosis but this view is no longer held

Granulomatosis infantiseptica appears to be more prevalent in Europe than in North America (Seeliger 1961) The disease is a generalized intra uterine infection occurring usually in the 3rd trimester with a high mortality rate for the fetus or child which is stillborn or born acutely ill It is characterized by extensive focal necrosis especially of the liver and the spleen more rarely of the lungs and the intestines Reiss *et al* (1951) first studied this form of the disease in Germany and since then over 200 cases have been seen in that country Meningitis occurs frequently in these cases In severe cases listeria can be isolated without difficulty from blood cerebrospinal fluid urine and other secretions Potel (1951) has observed that the meconium is generally positive perhaps due to swallowing of infected amniotic fluid Infants dying 2 or 3 days after birth often show a generalized petechial rash not unlike that of meningococcal septicemia

The pathogenesis of granulomatosis infantiseptica is not clearly understood but frequently listeria has been isolated post partum and sometimes antepartum from the mother s vagina and occasionally from her urine or blood Some of the mothers had noted at different times before delivery minor febrile illnesses on one or more occasions but none became seriously ill and most had a normal postpartum course Thus the mothers may have an inapparent infection manifested only by a rising agglutination titer Rapaport *et al* (1960) found that listeria infections were common among habitual aborters in one area of Israel but their findings have not been confirmed (Seeliger



Fig 1 Listeria keratoconjunctivitis 9 days after instillation of *L monocytogenes* showing edema corneal opacity and purulent exudate (After Gray Singh and Thorp Jr Abortion stillbirth early death of young in rabbits by *Listeria monocytogenes* 1 Ocular instillation Proc Soc Exp Biol Med *89* 163 169)

1963 Potel 1963) Cohen (1963) examined vaginal swabs from 561 pregnant women including 37 habitual aborters throughout pregnancy with completely negative results The consensus is that listeria is rarely concerned with habitual abortion in man

Diagnosis

The only absolute diagnosis depends on isolation and identification of the organism from a suspected case Cultures should be made from blood cerebrospinal fluid urine meconium placenta lochia milk or exudates at autopsies cultures should be made from all organs Tissues should be macerated in a few ml of sterile distilled water or nutrient broth not saline A portion of fluid specimen or macerated tissue should be

plated on tryptose or blood agar or on modified McBride's agar and the remainder stored at 4° C to be subcultured from time to time Girard and Sbarra (1963) have found phenylethanol agar with 0 05 per cent LiCl and 1 per cent glycine to be an adequately sensitive and selective medium After incubation for 24 hours at 37° C plates are examined using a hand lens or a scanning microscope using obliquely transmitted illumination Under these conditions colonies of *L monocytogenes* show a distinctive blue green color Care must be taken that colonies on blood agar are not confused with hemolytic streptococci or mistakenly discarded as diphtheroids because they grow on media containing potassium tellurite Examination of hanging drops for tumbling motility should be done Production of keratoconjunctivitis in the rabbit eye is of great value in identification The inoculation of experimental animals otherwise is not too reliable since most laboratory animals are susceptible to the natural disease Immunofluorescence has been used with varying success to demonstrate the presence of listeria in tissues

Serologic methods may be used as an aid to the diagnosis of listeriosis in man, but since the agglutination titer decreases rapidly after recovery it cannot serve reliably for late diagnosis One must also consider the fact that listeria has antigens in common with other pathogens especially staphylococci There is no bacteriologic evidence that antibody titers in normal healthy individuals or normal animals results from listeria infections These facts must be considered when utilizing the agglutination test as an aid to diagnosis The test should be done with H and O antigens of the 4 serotypes of *L monocytogenes* and with the autogenous strain Titers of over 1/200 are significant but a rising titer on consecutive serum samples is even more significant The serology of listerial infections has been reviewed by Seeliger (1961)

SPECIFIC THERAPY

In vitro studies by Linzenmeier and Seeliger (1954) indicate that, as a rule listeria is sensitive to penicillin streptomycin chloramphenicol, the tetracyclines and erythromycin but is resistant to sulfadiazine polymyxin and bacitracin These findings are borne out to some extent in the treatment of clinical listeriosis Sulfonamides have proved to be of little value Streptomycin is of limited value and the organisms may develop resistance to this antibiotic rather rapidly Cases have been treated successfully with combined penicillin and streptomycin and with tetracycline The present view is that tetracycline is the drug of choice Most cases of granulomatosis infantiseptica are fatal regardless of treatment and there have been so few reported cases of meningitis and septicemia that it is difficult, if not impossible to assess the claims made for various methods of treatment In all cases the strain isolated should be tested in vitro for its sensitivity to antibiotics

EPIDEMIOLOGY

Listeriosis is a disease with a very widespread distribution involving many unrelated and varied hosts with widely different food and living requirements, under every climatic condition from arctic to tropical The clinical pathologic and bacteriologic characteristics of listeriosis vary from one host to another, but there is no indication that this is due to difference in host species or in type of infecting bacteria The specific characters of strains of listeria isolated from all hosts in every country appear to be homogeneous apart from the fact that the 4 different serotypes differ in frequency in different regions No serotype is restricted to any particular host in fact strains isolated from cases not showing monocytosis as in cattle and generally in man nonetheless cause a characteristic monocytosis in rabbits

It is commonly assumed that human listeriosis is a disease with a low incidence and a high mortality rate This belief may be misleading because of the difficulty of isolating listeria whether from the sick or the normal individual Because of its widespread geographic distribution and its variety of hosts the organism in all probability has a high transmission rate and is able to persist for long periods of time in many individuals It

would appear that individual susceptibility or resistance is the principal determinant of disease, since animal experiments have shown that susceptibility to listeriosis can be altered by environment and general state of health

Ingestion of contaminated silage appears to play a role in animal outbreaks which have been studied by Palsson (1963) and Gray (1963) In addition Gray *et al* (1955b) have demonstrated that the addition of listeria to the drinking water of pregnant rabbits will result in abortion whereas nonpregnant or male animals are completely refractory to this method of exposure These findings are similar to those made in Germany where ingestion of contaminated cow s milk by a pregnant woman was associated with premature delivery of twins with granulomatosis from whom listeria was isolated (Potel 1953)

The oral route is not the only portal of entry since Gray *et al* (1955a) have reported intra uterine infection of rabbits following ocular instillation The genital route has been suspected as a source of infection of the human fetus but in many surveys of cervical or vaginal cultures *L monocytogenes* has never been demonstrated in normal women and in relatively few cases of abortion Listeriosis occurs as a venereal infection since the organism has been isolated from male urethral exudates by Wenkeback (1953) and more recently by Toaff *et al* (1962) from the semen of the male partners of several habitual aborters Respiratory exposure may also play a role particularly in encephalitis of ruminants and in septicemia and meningitis in man The organism has been isolated from throats of normal and encephalitic sheep but only twice from over 3 500 swabs of the human upper respiratory tract by Stanley (1950)

It is possible then that in naturally occurring human and animal listeriosis infection may result from ingestion inhalation or genital contamination Considerable evidence has accumulated in recent years which supports the first two hypotheses As brought out in the Second Symposium on Listeric Infection (1963) our knowledge of the epidemiology and the pathogenesis of listeriosis is very meager indeed In man the brunt of an outbreak may fall on the young in terms of both morbidity and mortality but the infection or carrier rate in children and adults remains unknown

ERYSIPELOTHRIX RHUSIOPATHIAE

History

Erysipelothrix rhusiopathiae is the etiologic agent of erysipeloid a not uncommon cutaneous infection in man which on occasion may be confused with streptococcal erysipelas Erysipelothrix infections also occur commonly in swine and are referred to as swine erysipelas A similar infection may occur in various fowl and is of some economic importance in turkey flocks The first member of this group was isolated by Koch in 1880 from the blood of mice and in 1886 by Loeffler from the cutaneous blood vessels of pigs that had died of swine erysipelas Rosenbach (1909) isolated *E rhusiopathiae* from human infections in 1886 and was the first to use the term erysipeloid for this disease in man While at one time strains of human porcine and murine origin were considered separately it is now generally held that these are almost identical variants of the single species *E rhusiopathiae*

There are some similarities between *E rhusiopathiae* and *L monocytogenes* which have led to both organisms being placed in the genus *Erysipelothrix* However this proposal is not widely accepted in North America since there are differences in both motility and pathogenicity between the two organisms and since there appears to be no antigenic relationship between them Woodbine (1950) has reviewed the bacteriology and the chemotherapy of erysipelothrix

Morphology and Cultivation

E rhusiopathiae is a gram positive non sporulating rod in which no capsule has been demonstrated It is micro aerophilic but will grow either aerobically or anaerobically Like listeria it occurs in a smooth and a rough form but differs from listeria in being nonmotile Smooth phase cells are small straight or slightly curved rods with rounded ends measuring 0 8 to 2 5 microns in length

and 0 3 micron in width and arranged singly, in small clusters or in short chains In the rough form long filaments of up to 60 microns or more in length predominate and long chains of bacilli may also be seen

E rhusiopathiae grows poorly on simple media but growth is improved by the addition of glucose and serum Smooth forms grow better at 33° C while at 37° C growth of the rough variant is favored After incubation for 24 hours at 37° C smooth phase colonies are round, glistening water clear and up to 0 1 mm in diameter On further incubation the colonies show little or no increase in size In the rough form the colonies are larger and flatter with a matte surface and a fimbriate edge which make them not unlike minute anthrax colonies The formation of a lateral outgrowth in gelatin stab cultures gives rise to the characteristic lamp brush appearance which when present is a diagnostic feature although it is not a consistent finding Gelatin is not liquefied Most smooth strains of erysipelothrix produce partial or alpha hemolysis around deep colonies in blood agar but a soluble hemolysin is not formed

Erysipelothrix is considerably less active biochemically than listeria It usually forms acid from lactose glucose galactose and fructose while mannose and maltose give inconsistent results (Byrne *et al* 1952) There is considerable variation in the fermentation pattern depending on the medium and the indicator used White and Schuman (1961) have found Andrade s base serum medium most dependable Erysipelothrix does not ferment rhamnose, mannitol sucrose dextrin or salicin The reactions for indole methyl red Vogues Proskauer and catalase are all negative Fuzi (1963) found that listeria strains are regularly very sensitive to neomycin while the erysipelothrix strains tested were all highly resistant

Antigenic Structure

Erysipelothrix and listeria are antigenically distinct Watts (1940) recognized 2 distinct antigenic types of erysipelothrix He found that each group possesses a heat stable specific antigen and 2 heat labile antigens present in different proportions in the 2 groups and responsible for cross agglutination Gledhill (1945) found both heat-stable and heat labile antigens, the former allowing 4 antigenic types to be distinguished Recent studies indicate that the heat stable fraction is a polypeptide (White and Kalf 1961) Both polypeptides and nucleoproteins were found by Truszczynski (1961) to be type specific antigens while species specific antigens were mainly nucleoproteins Two bacteriophages isolated by Brill and Politynska (1961) are specific for strains of serotype A which are all sensitive Ultraviolet irradiation revealed latent infection with Type A phages in the type B strains studied

Resistance

Exposure to moist heat for 15 minutes at 55° C will kill most strains of erysipelothrix The organism is resistant to salting pickling and smoking surviving in such treated meat for 1 to 3 months It may also remain viable and virulent for months in putrifying infected carcasses although it is very susceptible to drying. The organism will survive for 4 or 5 days in drinking water and for 12 to 14 days in sewage

Distribution and Range of Pathogenicity

E rhusiopathiae has a wide range of pathogenicity for animals under natural conditions and has a world wide distribution Natural infections have been reported in man swine sheep mice cattle horses and various species of domestic fowl Mice and pigeons are highly susceptible to experimental infection but not guinea pigs, although the latter are susceptible to listeria

Pathogenesis and Symptomatology

While erysipelothrix can produce a generalized septicemia with focal necrosis of liver and spleen in susceptible animals, it differs from listeria in manifesting a predilection for the skin the endocardium and joints

Infection in swine is commonly seen in 3 different forms a mild form known as red fever diamond skin disease or swine erysipelas" in which skin involvement predominates an acute severe form of septicemia and a chronic form characterized by arthritic symptoms The skin appears to be the preferred site for primary invasion in swine (Spencer, 1954) Experimentally

only oral administration has been successful in producing the symptoms of natural infection Recovered animals could not be reinfected (Rowsell 1955) Erysipelothrix infections of swine are often serious and fatal and may have considerable economic importance Infection of commercial turkey or chicken flocks is characterized primarily by involvement of the long bones from which positive cultures may be obtained The mortality rate in such flocks in a potential epidemic threat can be reduced materially by vaccination

The disease in man usually takes the form of a localized cutaneous infection known as Rosenbach s erysipeloid although septicemia with endocarditis and joint involvement may occur Interest in erysipeloid in North America dates from a report of Gilchrist (1904) in which 329 cases were seen in Baltimore 232 were individuals who had suffered abrasions while handling crabs The infection characteristically develops following an abrasion suffered while handling organic matter especially fish shellfish meat or poultry (Klauder 1938) Erysipeloid commonly appears as a mild cutaneous infection 1 to 7 days after inoculation usually in a finger or a thumb A small red sharply defined and slightly elevated spot appears at the site of inoculation and gradually spreads outward while the central area fades and takes on a purplish hue This is accompanied by edema and erythema and is followed by throbbing or tingling in the involved area A mild headache and malaise may be present. Suppuration does not occur and lymphangitis and lymphadenitis are irregular In about 6 per cent of patients arthritis may involve the injured area or adjacent joints Very rarely infection may become systemic and develop into an erysipelothrix septicemia which can be fatal In such an event there is seen a generalized purpuric and petechial rash resembling the rash of meningococcal septicemia A single attack apparently does not confer complete immunity in man as reports of reinfections are common

Diagnosis

A specific diagnosis of erysipeloid is based in part on clinical findings Characteristic appearance of the localized infection which almost invariably is on the hand and the absence of suppuration leukocytosis and systemic involvement all aid in differentiating human erysipeloid from cellulitis and streptococcal erysipelas However final diagnosis rests on the isolation of *E rhusiopathiae* from lesions Positive cultures are rarely obtained from material collected from swabs of a local lesion Biopsies should be taken and cultured for 24 hours in glucose broth followed by subculture on blood agar plates Rowsell (1958) held inoculated broth for 5 weeks at 5° C prior to plating a technic similar to that used for listeria Intraperitoneal injection of white mice with a suspension of biopsy material will yield a pure culture of erysipelothrix from heart s blood in 24 hours The organisms can be differentiated from listeria by biochemical reactions and by the fact that on instillation into the conjunctival sac of a rabbit listeria produces a severe keratoconjunctivitis whereas erysipelothrix causes a very mild conjunctivitis without keratitis In suspected cases of septicemia repeated blood cultures should be done No information is available regarding the positive diagnostic value of agglutination tests in man although in swine such tests have been used in diagnosis of chronic infection

Treatment

The evaluation of therapy in erysipelothrix infections is complicated by the fact that the disease is usually self limiting and runs a variable course with an average duration of about 3 weeks Both experimental and clinical studies have demonstrated that penicillin is the antibiotic of choice (Woodbine 1950) Streptomycin and sulfonamides alone have little effect Moynihan and Stovell (1954) found that penicillin tetracycline or a combination of penicillin and streptomycin were effective in turkeys experimentally infected

Epidemiology

Erysipeloid in man is primarily an occupational disease seen in individuals handling meat fish poultry or shellfish King (1946) summarized 115 cases treated at a London hospital and found that 85 of these were directly attributed to an animal source Abattoir workers and fish handlers predominated in a group of 100 cases studied by Klauder

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(1938) Outbreaks of the disease have been described in workers in a bone button factory and cases have occurred in veterinarians However erysipeloid also has been observed as a nonoccupational disease in sporadic cases

Although in man inoculation almost invariably takes place through broken skin in swine other portals of entry apparently are possible In experimental swine erysipelas Rowsell found that various routes of infection produced tonsillar carriers in 92 per cent of the pigs Tonsillar carrier rates of 11 to 31 per cent have been found in swine (Connell and Langford 1951 Rowsell 1958)

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32

Streptobacillus Moniliformis

DEFINITION

Streptobacillus moniliformis is an aerobic gram negative pleomorphic microorganism requiring blood serum or ascitic fluid for cultivation In the animal body and in young cultures grown in completely satisfactory media the morphology is more uniformly bacillary (2 to 4 microns in length) whereas in older cultures and in cultures in slightly unsatisfactory media the morphologic forms consist of filaments up to 40 microns in length and chains of bacilli or cocci Often these filaments show swellings 2 to 5 times their own width The words *Streptobacillus* and *moniliformis* constitute the most succinct description of the morphology of this organism A variant designated L_1 (L = Lister Institute) is produced The organism is a normal inhabitant of the nasopharynx of wild and laboratory rats It is highly pathogenic for mice nonpathogenic for guinea pigs rabbits and usually rats In man it is the cause of one type of rat bite fever and of a disease known as Haverhill fever or erythema arthriticum epidemicum which is characterized by fever rash and polyarthritis but is not necessarily acquired by the bite of a rat (Synonyms *Streptothrix muris ratti Actinomyces muris Asterococcus muris Haverhillia multiformis* etc)

HISTORY

The fact that rat bite fever may be caused by two different microorganisms *Spirillum minus* and *Streptobacillus moniliformis* and the extreme variation of the latter organism have led to a very confused literature The first case of rat bite fever was described by Wilcox in 1840 but the role of streptobacillus under various names was not discovered until 1914 and the years immediately ensuing The epidemics in Chester Pa (Place and Sutton 1934) and in Haverhill, Mass (Parker and Hudson 1926) have demonstrated that the bite of a rat is not essential for man s acquiring the infection For a good review of the disease and its causative organisms the reader is referred to the publication of Brown and Nunemaker (1942)

CULTIVATION AND BIOLOGIC PROPERTIES

One of the characteristics of *S moniliformis* is that it requires the presence of a natural body fluid for growth on artificial medium Tryptose phosphate broth and media of the infusion type are satisfactory basal media These require enriching with blood serum or ascitic fluid to a concentration of 10 to 30 per cent. The organisms grow better in media with a pH of about 7 6 Optimum growth takes place in an aerobic atmosphere at 37 C although some strains may grow better in an atmosphere of increased CO tension (Smith and Sampson 1960) Growth on solid medium is favored if the basal medium contains slightly less than the conventional 1 5 per cent agar and the atmosphere contains a high moisture

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formed back to the streptobacillus organism (Heilman 1941a) Compared with *S monil iformis* L_1 is less virulent if virulent at all for the mouse and it is a poorer immunizing antigen (Freundt 1956b) *S moniliformis* has been reported to produce acid without gas from glucose maltose levulose salicin starch and glycogen and to being inert to ward lactose sucrose mannitol xylose inositol inulin dulcitol arabinose raffinose sorbitol trehalose rhamnose and glycerol It is variable toward galactose The L_1 vari ant has fermentative activities identical with those of *S moniliformis* (Heilman 1941b) An interesting association of two micro organisms is the dependence of *Entamoeba histolytica* on its associated streptobacillus for metabolism of glucose (Loran *et al* 1956)

The view of Dienes (1939) is now gener ally accepted that the L_1 organism is a vari ant of *S moniliformis* An effort is being made (Bassermann *et al* 1957) to employ the term L with some consistency It is recommended that this term be reserved for a type of growth arising spontaneously or by stimulation which is characterized by a colony of special appearance showing a dense center and lighter periphery on media not containing the original stimulating sub stances Furthermore microorganisms com prising this L form must not assume the morphology of the parent culture Organisms in the L_1 colonies grow into the agar medium so that they cannot be transferred by the usual bacteriologic technics using an inocu lating needle The agar block transfer tech nics of Klieneberger (1935) give the best results

The organisms are extremely fragile and soft Young colonies and the central mass of well developed colonies consist of small forms which often appear as tiny bipolar stained bacilli These forms are transformed

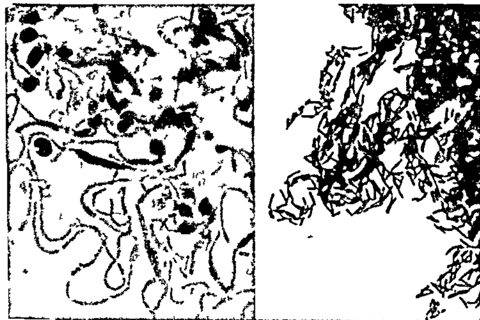


FIG 2 (*Left*) *Streptobacillus moniliformis* colony (24 hour agar culture in situ with methylene blue) Note the irregular wavy filaments containing deeply stained pear shaped swellings Magnification × 1855

FIG 3 (*Right*) Broth culture of *Streptobacillus moniliformis* (Gram stain of 24-hour growth) Note the lack of pleomorphism Magnification × 1855 (Dr Louis Dienes)

content In a few instances it has been found possible to replace the serum component in the medium by starch (Heilman, 1941b) glycogen or dextrin (Dumoff and Duffy 1951)

In fluid cultures growth usually takes place in the form of fluff balls or bread crumbs on the bottom of the tube or on the surface of the sedimented red blood cells The supernatant fluid may remain clear and devoid of organisms A fluff ball should be removed with a pipette for transfer or microscopic examination Giemsa s and Wayson s stains are more satisfactory than Gram s technic for demonstrating the microorganisms Fluid cultures may require daily transfer in order to maintain the viability of the strain

Another characteristic of the organism is its extreme pleomorphism in microscopic preparations The cells vary from fairly uniform small slender rods to long slender filaments which are often irregularly fragmented and are said to resemble the dot-dash appearance of the Morse code In addition they show circular to spindle shaped swellings (Fig 1)

On solid medium growth takes place in 2 to 3 days more slowly than in liquid media and the organisms are viable for a longer period i e , up to 1 week Individual colonies are raised and granular and may become as large as 5 mm in diameter Beneath or adjacent to the colonies of streptobacillus the microscopic L_1 colonies may develop Organisms in the L_1 colonies are much more resistant to penicillin than are those in the streptobacillus colonies, consequently penicillin can be used to isolate the L_1 component The L_1 organism can be obtained and maintained in pure culture and can be trans



FIG 1 *Streptobacillus moniliformis* and pleuropneumonialike organism L (24-hour culture on ascitic fluid agar stained in situ with methylene blue Azure II) Magnification × 112 (Dr Cynthia H Pierce)

formed back to the streptobacillus organism (Heilman 1941a) Compared with *S monil iformis* L_1 is less virulent if virulent at all for the mouse and it is a poorer immunizing antigen (Freundt 1956b) *S moniliformis* has been reported to produce acid without gas from glucose maltose levulose salicin starch and glycogen and to being inert to ward lactose sucrose mannitol xylose inositol inulin dulcitol arabinose raffinose sorbitol trehalose rhamnose and glycerol It is variable toward galactose The L_1 vari ant has fermentative activities identical with those of *S moniliformis* (Heilman 1941b) An interesting association of two micro organisms is the dependence of *Entamoeba histolytica* on its associated streptobacillus for metabolism of glucose (Loran *et al* 1956)

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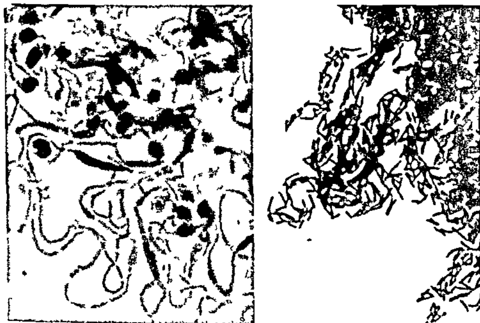


FIG 2 (*Left*) *Streptobacillus moniliformis* colony (24-hour agar culture in situ with methylene blue) Note the irregular wavy filaments containing deeply stained pear shaped swellings Magnification × 1855

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at the surface and at the periphery of the colonies by gradual swelling into large round forms The morphology of the organ isms in the L_1 colonies is so similar to that of pleuropneumonialike organisms that, with out knowing the origin of a culture after it has lost its ability to return to the strepto bacillus it would be impossible to differ entiate between the two on the basis of morphology (Dienes 1947)

The production of L colonies from bacteria and the reversion to the bacillary phase have been interpreted by Klieneberger-Nobel (1951) as a process of regeneration in bac teria initiated by a fusion of certain elements In some cases when the multiplication rate of bacteria is disturbed during a state of vigorous growth the bacteria react by trans forming into the L phase in which the organisms can better withstand the adverse conditions Organisms in the L phase mul tiply by segmentation they eventually break up into small granules which are capable of germination and in some cases are filterable In addition the L forms show differences in food requirements and in metabolism such as resistance to penicillin and other chemicals (Edward 1953)

EPIDEMIOLOGY

Infections with *S moniliformis* are usually acquired through the bite of a rodent (rat squirrel weasel) Frequently there is no known history of rodent bite or animal con tact but there may be a history of trauma In rare instances the infection may be ac quired from contaminated food

S moniliformis is a normal inhabitant of the respiratory tract of laboratory and wild rats Being present particularly in the upper portion of the respiratory tract the naso pharynx the organisms are transmitted read ily during the act of biting by the animal In rats there may be involvement of the lungs and the middle ears but generally rats are refractory to infection (Strangeways 1933) Investigators have failed to isolate the organisms from the mouths of normal healthy mice but occasionally an epizootic disease resulting from *S moniliformis* occurs among laboratory (Freundt 1956a) and wild mice (Williams 1941) There is no reported case of rat bite fever in man as a result of the bite of a mouse but man has acquired the infection by handling mice (Levaditi *et al* 1925) Squirrels and weasels have been known to transmit the infection An infection among turkeys which were housed in the vicinity of rats has been re ported (Boyer *et al*, 1958)

Cases have been reported in which there is no history of a rodent bite or contact with animals such as that reported by Sprecher and Copeland (1947) In one instance the infection developed after a fall (Levey and Levey 1948) and in another instance it may have been associated with trauma (Hazard and Goodkin 1932)

Contaminated food may be the source of sporadic outbreaks or isolated cases The epidemics in Chester Pa in 1925 and in Haverhill Mass in 1926 are suspected of having been milk borne (Place and Sutton 1934) and the case reported by Oeding and Pedersen (1950) is also ascribed to con tamination of food or milk

PATHOGENESIS

From the clinical features the pathogenesis of rat bite fever becomes obvious Following the inoculation of a susceptible individual with *S moniliformis* through a bite wound there is a proliferation of the organisms at the site of the wound extension to the neigh boring lymphatics and lymph nodes and eventually an invasion of the bloodstream accompanied by severe toxic symptoms En docarditis either acute or subacute is a seri ous complication (Petersen *et al* 1950 Hamburger and Knowles 1953) The dura tion of the disease may vary from a period of a few days to one of several weeks In un treated cases the mortality rate is about 10 per cent

In the case of a food borne infection the portal of entry of the organisms is thought to be the intestines gastrointestinal symp toms are common However the rapid loss of viability of streptobacillus cultures in a slightly acid environment casts serious doubt on this view The oral region or the throat might also be considered as a possible portal of entry throat soreness is a frequent symptom

In a comprehensive study (Freundt 1956a) mice were readily infected by sub cutaneous intraperitoneal intravenous and intranasal routes as well as by feeding and less readily by contact with rats The mice were infected but not easily through the conjunctival sac in this particular study In the feeding experiments it was definitely shown that invasion of the host by the micro organisms did not take place through the intestines but rather through the mouth or the throat Mice with different genetic back grounds show different degrees of suscep tibility (Mackie *et al* 1933)

In the acute stage the infection is likely to be of the septic type In the chronic type where the host may show greater resistance there is a greater tendency toward abscess formation and joint involvement In experi mental infections in the developing chick embryo the organisms invaded the blood stream and localized almost exclusively in the synovial lining of the joints (Buddingh 1944) In the early stages of the develop ment of the joint lesions *S moniliformis* was found to behave as a facultative intracellular parasite within the cytoplasm of the synovial lining cells While the rat is regarded gener ally as being fairly resistant to infection by *S moniliformis* one strain has been found to be pathogenic for the laboratory rat (Lerner and Silverstein 1957) The animals showed an osteoarthritis which was often accompanied by an osteomyelitis periostitis arthritis and periarthritis

S moniliformis is unusual in that it has been demonstrated to produce in vivo the L_1 variant which is endowed with properties of greater resistance and this variant is capable of reverting to streptobacillus

DIAGNOSIS

Rat bite fever resulting from *S monili formis* may vary in its symptomatology in individual cases but the clinical picture is sufficiently characteristic so as not to be overlooked readily (Blake 1916) A typical case presents usually but not always the history of rat bite a latent incubation period of variable length with subsequent nonsup purative inflammatory reaction at the site of the wound, lymphangitis enlarged lymph nodes severe chill at onset high fever of the relapsing type leukocytosis intense mus cular pains nervous symptoms and bluish red exanthem. Endocarditis and arthritis may be complications and a false positive Was sermann reaction may occur

It is important to attempt to confirm the tentative clinical diagnosis of rat bite fever by demonstrating the causative organism This may be either *Spirillum minus* or *S moniliformis* *S minus* is demonstrated by animal inoculation whereas *S moniliformis* is demonstrated by cultural methods

S moniliformis may be isolated in routine blood cultures from patients if a good cul ture medium is employed such as infusion broth or tryptose phosphate broth and a sufficient amount of the patient s blood is added to each container of broth to give a concentration of nearly 20 per cent When growth occurs it is found on the surface of the sedimented blood cells and on the in clined surface of culture tubes this growth resembles bread crumbs fluff balls or cot ton balls in its gross appearance The organism has also been isolated from the fluid aspirated from involved joints

An agglutinin titer of 1 80 or greater with streptobacillus antigen is considered as in dicative of infection with that organism The specific streptobacillus agglutinins may ap pear as early as 10 days or as late as 2½ months after the bite may attain their maxi mum titer in 1 to 3 months after the bite and may disappear within 5 months or persist for over 2 years (Brown and Nune maker 1942)

TREATMENT

Penicillin inhibits the growth of *S monili formis* in vitro protects mice against experi mental infections (Heilman and Herrell 1944) and appears to be effective in the treatment of some cases of the natural in fection in man (Altemeier *et al* 1957 Wheeler 1945) Streptomycin was found to be effective in the treatment of a natural infection which did not respond to penicillin (Sprecher and Copeland 1947) and to be more effective than penicillin in the treat ment of mice after joint involvement had developed (Levey and Levey 1948) The

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L_1 variant along with pleuropneumonialike organisms, is resistant to penicillin, and although penicillin may free the body of the *S moniliformis*, the L_1 organism may persist and a broad spectrum antibiotic such as chlortetracycline must be employed to bring about its elimination from the body (Dolman *et al* 1947)

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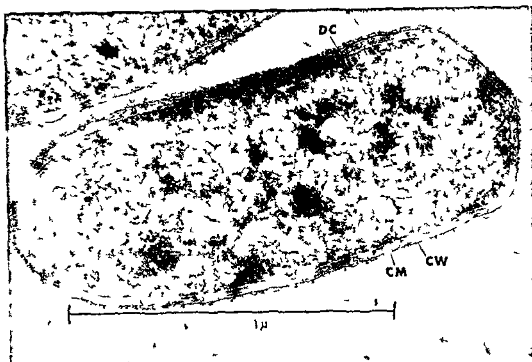


FIG 1 An electron micrograph of a strain of bacteroides showing the general cell morphology and cell wall (CW) cytoplasmic membrane (CM) and dense crust (DC) (Bladen H A and Waters J F J Bact *86* 1340)

implicated bacteroides in diseases of man and animals and perhaps the most pathogenic In lesions the organisms may not be very pleomorphic and may resemble *H influenzae* However they may be very pleomorphic in cultures showing characteristic swellings—spheroid bodies up to 4 microns in diameter—in the middle or at the end of a rod or filament in certain cases the entire cell may be a spheroid body These structures were pictured in photographs by Dienes and Smith (1944) and in early electron micrographs by Smith *et al* (1948) Dienes (1942) stated that all strains of *B funduliformis* isolated from pathologic lesions produced numerous large bodies Although these degenerated in most cultures without further development Dienes reported that they were capable of germinating as small granular forms which developed into the L type colonies rather than into bacteria of regular shape The L type colonies were isolated in pure culture from one strain and maintained through 12 consecutive transfers (Dienes 1941) Another strain of *B funduliformis* swelled into large round bodies but produced no L-type colonies In another study Dienes and Smith (1942) stated that the large round bodies may (1) elongate to a bacillary form (2) take on an irregular angular shape and by division fractionate into 2 to 4 parts which develop into regular bacterial cells (3) occasionally show regular shaped bacteria developing within them which are freed when the large body disintegrates or (4) in the majority of cases increase in size and density develop many prominences which develop bizarre shaped branching filaments producing regular bacilli by fractionation The large bodies germinate only in spontaneously pleomorphic strains and not in cultures made pleomorphic by toxic influences (Dienes and Smith 1944)

B melaninogenicus is a good example of the confusing background which may be associated with organisms of the bacteroides

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33

Bacteroides

The ill defined *Bacteroides* group is made up of organisms which usually are extremely numerous in the stools of adults and oc casionally are associated with pathologic processes in man and animals

Bacteroides are gram negative non spore forming strictly anaerobic rods usu ally pleomorphic with rounded or pointed ends they vary in size the smallest forms being filterable They may be motile or non motile Frequently body fluids are required for growth They are found primarily in the intestinal tract and on the mucous mem branes of warm blooded animals

HISTORY AND GENERAL CHARACTERISTICS

Recognition of bacteroides was delayed by the attention given to other anaerobes in particular the clostridia Awareness of their role in infectious processes of man began in 1897 and 1898 with the findings of Veillon and Zuber who isolated these organisms from brain abscesses lung gangrene peritonitis etc The report by Eggerth and Gagnon (1933) that bacteroides were the pre dominating organisms in 91 per cent of the stools of adults focused attention on them Dack (1940) reported that nearly 4 per cent of 5 180 specimens submitted for bac teriologic examination by a department of surgery contained non spore forming anaer obes over half of which appeared to be bacteroides The history of the bacteroides group is complicated by the multiplicity of names given to the same organism

Electron micrographs of bacteroides cells show a rigid cell wall surrounding the cyto plasmic membrane (Bladen 1963, Bladen and Waters 1963) (Fig 1) The general morphologic and biologic properties are illus trated best by a description of representative strains which are encountered most fre quently Many of the strains in this group have been studied so little that only general conditions for cultivation are given in the section on diagnosis

Bacteroides fragilis is commonly taken as the representative example of the large group of pleomorphic nonmotile non spore form ing anaerobic rods It receives its name from the tendency of the colonies to autolyze It metabolizes a variety of carbohydrates with the production of acid but the reactions are variable It frequently infects man but the infections are less acute and less severe than those produced by *B funduliformis*

B funduliformis is the most extensively studied member of the bacteroides group It is listed in the 7th edition of Bergey s Manual as *Sphaerophorus necrophorus* It is typical of several highly pleomorphic strictly anaerobic gram negative rods described under a variety of names The term *Bac teroides funduliformis* seems preferable be cause it has been used so extensively through out the scientific literature and because *Sphaerophorus* is an illegitimate homonym

B funduliformis is the most frequently

nuclear cell response and the bacteroides may be intracellular and extracellular They may be mistaken for *H influenzae* in gram stained smears The meningitis may be localized in the vicinity of the infected ear but more frequently it is extensive over the base of the brain The facial nerve may be involved Sinus thrombosis may be a complication giving rise to invasion of the blood stream and septic infarcts in the lungs

DIAGNOSIS

Two important aspects of the laboratory diagnosis of infections caused by bacteroides are that the culture medium usually needs to be enriched with blood blood serum ascitic fluid or tissue furthermore the cultures require anaerobic conditions for growth When the patient shows a septic condition or there are abscesses with foul smelling pus or there are foul smelling discharges bacteroides should be considered Frequently the symptoms do not reflect the seriousness of the infection Some of the bacteroides produce gas hence differentiation from gas gangrene must be made (Biegelman and Rantz 1949) Thioglycollate medium or other general purpose media such as glucose broth heart infusion broth etc containing brain or muscle tissue are satisfactory for primary isolation from blood or other sources If there is a tall column of liquid in the tubes and the dissolved air has been expelled by heating and rapid cooling prior to inoculation a petrolatum seal is unnecessary However if it is desired to detect the production of gas then a seal is advisable Blood agar is a fairly satisfactory solid medium for isolating and studying the organisms

It is advisable not to leave the cultures exposed unnecessarily to the ordinary atmosphere since oxygen is toxic to some strains If the organisms stain poorly by Gram s method or with methylene blue carbol fuchsin should be tried The morphology varies depending on the conditions of cultivation but together with growth requirements it may be sufficient for a broad differentiation within the group Reactions to test carbohydrates and protein substrates may be used for finer classification but this usually is unnecessary in most cases Little work has been done on antigenic structure preliminary studies indicate that these organisms constitute a heterogeneous group

TREATMENT

From the few reports on the drug sensitivities it appears that bacteroides are usually susceptible to chloramphenicol chlortetracycline and oxytetracycline They are occasionally resistant to bacitracin and generally resistant to dihydrostreptomycin streptomycin polymyxin, erythromycin and penicillin (Foley, 1947 Garrod 1955 Mc Vay and Sprunt 1952 Stevens and Harrison 1958) Two strains of *B fragilis* among 23 tested were found by Garrod to produce penicillinase

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Eggerth A H and Gagnon B H 1933

group It was originally described by Oliver and Wherry (1921) who isolated it from the upper respiratory tract, the urinary tract and surgical wounds It was so named because of the black pigment produced on blood agar and thought to be melanin Schwabacher *et al* (1947) showed that the pigment was in reality hematin, not melanin united with a bacterial protein to form a parahematin, they suggested that the name be changed to *Fusiformis nigrescens* They also stated that since the organisms produce acid in basal media without carbohydrates fermentation tests were unsatisfactory According to Garrod (1955) the pigment cannot be produced until hemolysis has occurred and is not seen well in less than 5 days

It is often stated that this organism is not pathogenic for common laboratory animals but Weiss (1943) who isolated it from 45 surgical patients discovered that if it was injected subcutaneously within a few days after cultivation, edema and inflammation were produced Intradermal injection into rabbits produced local inflammation dermonecrosis and occasionally death Production of fibrinolysin was demonstrated by Weiss in 1937 All 22 strains studied by Garrod (1955) were very sensitive to penicillin

Fusobacterium fusiforme is typical of the spindle shaped organisms which have been given generic status although not all organisms which are spindle shaped are *Fusobacterium* The organisms are usually slender rods either straight or slightly curved with tapering ends At least one species is motile Filaments and branching forms may be produced and are shown in electron micrographs published by Mudd *et al* (1942) and by Hampp *et al* (1960) The organisms possess a rigid cell wall typical of bacterial cells In lesions they are frequently associated with spiralled organisms for example in Vincent s gingivitis

The organisms are commonly present in the mouth and the throat of normal persons They are frequently found in cases of stomatitis (trench mouth Vincent s angina) ulcerative processes of the throat, the colon and the genitalia and lung abscess Because the organisms are relatively sensitive to penicillin Vincent s angina is amenable to penicillin therapy

Dialister In a series of papers starting in 1921, Olitzky and Gates described very small anaerobic, gram negative rods which could be isolated from the Berkefeld filtrates of nasopharyngeal secretions They designated the organisms *Bacterium pneumosintes* but these are now placed in the genus *Dialister*

PATHOGENESIS

There is no evidence that the bacteroides play a primary role in the production of disease However in injured or diseased tissues they may be responsible for serious and persistent infection Since bacteroides are commonly present on the mucous membranes, tissue infection probably originates as a result of their penetrating the mucous membranes of the nose the middle ear the pharynx the tonsils the urinary tract or the intestinal tract They may also penetrate through the skin in rare instances Local abscesses may be formed or a bacteremia produced followed by metastatic abscesses Of 11 patients from whom bacteroides were cultured from the blood only 1 survived and this patient received 347 Gm of sulfanilamide during 29 days of therapy (Brown *et al* 1941) Patients with bacteremia show acute toxic condition chills and fever, extreme weakness sweating and leukocytosis Infected embolic lesions occur in the lungs and in various other regions of the circulatory system The abscesses show a tendency to spread rather than to be walled off The pus is usually cream-colored and foul smelling The central portion of abscesses and infected thrombi frequently show liquefaction (Thompson and Beaver 1932) Icterus is frequently observed when the portal of entry is the gastrointestinal tract In the series of 35 patients studied by McVay and Sprunt (1952) 21 were critically ill and 11 died Bacteroides appeared to be the primary cause of death in 9 patients and secondary invaders in 2 cases

Peritonitis following salpingitis has been observed to resemble gonococcal peritonitis (Smith and Ropes 1945)

Meningitis and brain abscess may follow a chronic suppurative otitis media (Smith *et al* 1944) and is more prevalent in adults than in children There is a polymorpho

nuclear cell response and the bacteroides may be intracellular and extracellular They may be mistaken for *H influenzae* in gram stained smears The meningitis may be localized in the vicinity of the infected ear but more frequently it is extensive over the base of the brain The facial nerve may be involved Sinus thrombosis may be a complication giving rise to invasion of the blood stream and septic infarcts in the lungs

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34

The Bartonella Group

Bartonella bacilliformis the cause of Carrion s disease in man and micro-organ isms native to other animals of the genera *Haemobartonella* and *Eperythrozoon* will be considered together here They have been grouped because of common characteristics All are insect transmitted proved or pre sumptive usually cause acute primary in fectious febrile anemias in their vertebrate hosts have a characteristic position on the erythrocytes and are carried as asymptomatic infections for long periods of time Despite these similarities electron microscopy indi cates fundamental differences of structure between the two groups *Bartonella bacilli formis* is an undoubted bacterium the nature of the animal pathogens is still in doubt

BARTONELLA BACILLIFORMIS

Definition

Bartonella bacilliformis produces unique and striking clinical conditions which define the organism better than the characteristics thus far discovered in vitro In addition to nonclinical asymptomatic infections of pre sumed epidemiologic importance *B bacilli formis* causes two very different and appar ently unrelated conditions (1) Oroya fever a febrile anemia and (2) Verruga peruana a benign skin eruption Each form has a distinctive pathologic substratum and the two are linked immunologically In Oroya fever bartonella is found on the erythrocytes In both diseases it occurs within fixed tissue cells notably those of the reticuloendothelial system On the red cells it is unmistakable and no other human parasite resembles it even slightly (Figs 1 and 2) In the tissues it is intracellular and during Oroya fever multiplies within the cytoplasm of vascular endothelial cells grouped in rounded masses or as isolated elements (Fig 1 3) A poly morphic bacillus often flagellated in culture *B bacilliformis* can be maintained by un limited serial transfer on media containing serum Living cells are not required how ever when grown in tissue culture develop ment is both intracytoplasmic and extra cellular The microorganism occurs in nature in phlebotomus and in man

History

This is in part a record of scientific documentation for one of the most improb able of New World marvels The excellent monograph of Odriozola (1898) drew at tention to a disease believed to occur only in western South America There it was re stricted to certain mountain valleys and con tracted only at night This unique disease was said to exist in two forms clinically unre lated One an anemia frequently fatal occurring at times in epidemics was often so fulminant that blacks became whites in a few days The other form benign was distinguished by a skin eruption the like of which was unknown outside the Andean valleys To the surprise of the scientific com munity all of these items have proved to be substantially correct

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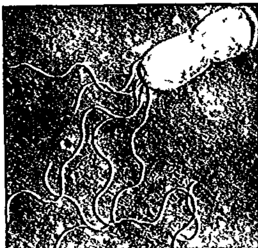


FIG 2 *Bartonella bacilliformis* from an 8 day culture Hemolyzed palladium shadowed Note unipolar flagella cell membrane and commencement of binary division Magnification × 19 000 (Peters D and Wigand R 1952 Neue Untersuchungen uber Bartonella bacilliformis I Morphologie der Kulturform Z Tropenmed Parasit *3* 313 326)

Verruga was in all probability recorded by the pre-Columbian Indians before the regional introduction of writing in the form of anthropomorphic jugs (huacos) in which characteristic lesions are represented (d Harcourt 1939 Mazzini 1934)—evidence which agrees with early writings of the Spaniards derived from Indian sources The anemic form did not attract much attention until 1870 when an epidemic caused an estimated 7 000 deaths In 1885 Carrion wishing to define symptoms in the pre eruptive stage of verruga was inoculated with verruga material and died of Oroya fever 39 days later strongly suggesting the etiologic unity of the two conditions (Medina *et al* 1925) The microorganism was reported by Barton in 1909

Experimental work speeded in 1910 with the discovery of the susceptibility of *Macaca mulatta* to verruga (Jadassohn and Seiffert) The relationship of Barton s organism to Oroya fever was confirmed in 1915 by Strong Tyzzer Brues Sellards and Gastiaburu who named it described the pathology of Oroya fever and discovered the pathognomonic lesion found therein Noguchi and Battistini in 1926 cultivated the organism described the relation of *B bacilliformis* to verruga thus producing experimental evidence supporting the unitarian etiology and Noguchi subsequently gave detailed descriptions of the cultural and the biologic charac

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FIG 1 All blood films were stained with Giemsa s fluid after fixation either by the May Grunwald mixture or by methyl alcohol (Weinman D 1944 Infectious anemias due to bartonella and related red cell parasites Tr Am Philosophical Soc *33* 243 350)

(1 and 2) *Bartonella bacilliformis* in blood films of two fatal cases of Oroya fever In (1) the infection is intense the bartonellae parasitize not only the erythrocytes but are also found within monocytes (Original)

(2) The straight and curved rods chains dots and rings illustrate the great morphologic range of *B bacilliformis* (Original magnification as in (1))

(3) Section of an Oroya fever lymph node Development of *B bacilliformis* in distended endothelial cells lining the vein The intracytoplasmic distribution in rounded clumps is distinct in the heavily infected cells (Redrawn from C Uribe)

(4) Section of a human verruga Regaud fixation Giemsa stain The blood capillaries are numerous and the proliferated endothelial cells conspicuous *B bacilliformis* stains red is very evident and is often located distinctly within the cytoplasm of endothelial cells Note that, in spite of the numbers of microorganisms in the tissues none appear on the erythrocytes (Original)

(5) *Haemobartonella microti* in blood films of splenectomized mouse. Variation in shape is extreme Rods coccoids and rings occur both singly and in various combinations constituting bow forms filaments which contain rings or coccoids rows of rings and coccoids etc (Redrawn from E E Tyzzer)

(6) *Eperythro oon coccoides* in the blood of a splenectomized white mouse An intense infection in which as is customary rings preponderate The resemblance to certain forms of *B bacilliformis* is quite noticeable (Original)

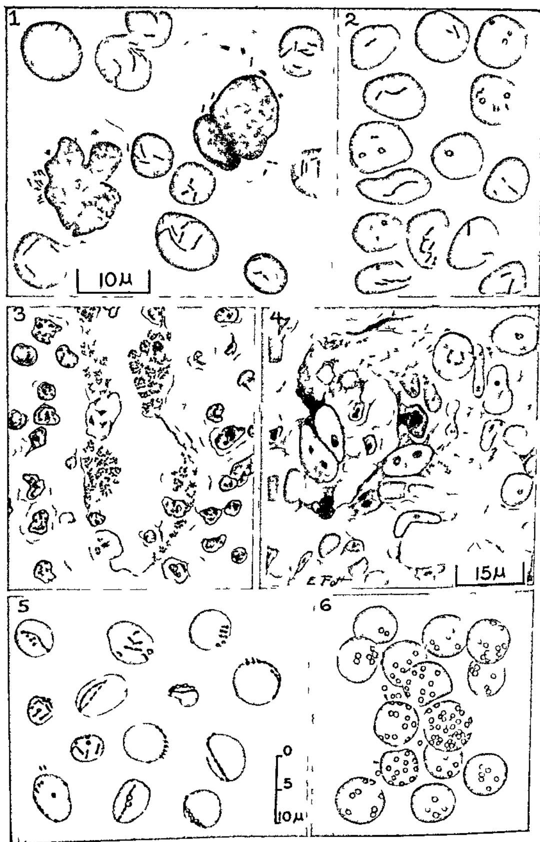


FIGURE 1 (*Caption on facing page*)

to 10 in number vary in length from 3 to 10 microns and have a diameter of about 20 millimicrons and undulation phases of 0 95 micron (Noguchi Peters and Wigand 1952) As noted above flagella have not been demonstrated with certainty on the epierythrocytic forms and aflagellar strains exist A definite cell membrane can be demonstrated by appropriate technics applied to either blood or culture forms (Fig 2) In this respect bartonella resembles other gram negative bacteria but differs from apparently closely related members of the bartonella group (Peters and Wigand 1955) Bartonella does not traverse the usual bacteriologic filters (e g Berkefeld N)

The most favorable temperature for the semisolid cultures is 28 C The optimum pH is 7 8 with a range from 6 8 to 8 4 Bartonella is an obligate aerobe It produces neither acid nor gas with any of the numerous carbohydrates tested does not liquefy gelatin and has no action on lead acetate All attempts to isolate a hemolysin have failed it does not hemolyze red cells in culture or even localize on or about the erythrocytes when they are added to a semisolid medium Pathogenicity is retained in semisolid leptospira medium for several years at least whereas blood agar slants are generally less favorable in this respect Only media containing blood have been successful serum or plasma has appeared to be essential and hemoglobin is favorable because it supplies hemin Enrichment of media with glutathione and ascorbic acid may give heavier growth (Geiman 1941) Bartonella is sensitive in vivo to penicillin streptomycin chloramphenicol and tetracycline organic arsenicals are relatively inactive It is a facultative intracellular parasite when grown in tissue culture and then reproduces the appearance seen in necropsy or biopsy material Cultures may be stored at −70 C in semisolid medium These are viable for years a strain obtained in 1950 thawed and subcultured several times in the interval grew after transfer in 1961

Range of Pathogenicity

Bartonella manifests full pathogenicity for man only In certain monkeys and apes notably *Macaca mulatta* (*M rhesus*) verrugas may be produced with great regularity and passed in series but Oroya fever cannot be reproduced in animals at will and splenectomy has not made it possible to produce predictable Oroya fever infections in monkeys (Noguchi Weinman and Pinkerton 1937a Wigand and Weyer 1953) Usual laboratory animals including the developing chick embryo are not satisfactory even for verruga production The route of inoculation and the nature of the inoculum are of prime importance in determining the character of the infection Satisfactory inocula such as cultures verrugas of either human or simian origin or tissue from Oroya fever patients injected into the skin or subcutaneous tissue of *M mulatta* produce a local verruga which is sometimes multiple but very rarely generalized Oroya fever blood almost never produces verrugas in monkeys yet infected lymph nodes from an Oroya fever patient or cultures made from Oroya fever blood will produce verrugas in almost every instance when appropriately injected This is a puzzling feature and is not explicable by quantitative factors alone

Pathology and Pathogenesis

In Oroya fever the body is pale and somewhat icteric the liver enlarged the spleen frequently so and often infarcted and the lymph nodes usually enlarged are often hemorrhagic

The microscopic appearance is distinctive and results from the development of masses of bartonella within the cytoplasm of cells lining the blood and the lymph capillaries The growth reaches an extreme degree causes the cytoplasm to expand to many times its normal width and to bulge into the lumen of the vessel The entire cytoplasm may be filled with microorganisms which tend to form rounded masses or clumps (Fig 1 3) Infected cells are particularly abundant in lymph nodes and also occur in the liver the spleen bone marrow the kidney the adrenals the pancreas more rarely in the skin the heart and the lung Gross lesions such as the splenic infarcts may follow vascular occlusion by the swollen endothelium

Verruga peruana differs as much in microscopic appearance from Oroya fever as do

teristics of the cultivated organism These results not duplicated at first in other laboratories, were finally fully confirmed by the Harvard 1937 Expedition to Peru (Weinman and Pinkerton Pinkerton and Weinman) These authors noted the prevalence of non clinical or latent infections in man and suggested the importance of the human reservoir Taken in conjunction with the prior discovery of phlebotomus as the vector by Shannon and by Noguchi this has given a much clearer picture of the conditions for perpetuation and extension of the disease Hertig (1938) re identified the species of phlebotomus concerned and with Fisher (1945), indicated that control might be achieved with DDT a result which has since been accomplished in a number of areas Excellent morphologic detail in electron microscope preparations was obtained by Peters and Wigand (1952 1955) the bearing of this and cytochemical and serologic findings on classification within the bartonella group is discussed fully by Wigand (1958) The major therapeutic problem i e, the treatment of Oroya fever appears to have been solved by the use of antibiotics A monograph by Weinman (1944) treats of the human and the animal micro organisms in great detail

General Characteristics

Bartonellosis is a microbiologist s curio since it is unique not only in the duality of its clinical forms a duality expressed too in the pathology of each but it is also distinctive in the interrelation and the opposition between the two forms These latter characteristics reflect in all probability successive immunologic states of the same individual It is one of the most perfect examples of a geographically restricted bacteriologic infection being exclusively American exclusively tropical exclusively Andean and proved to exist only in mountainous regions of Peru Colombia and Ecuador

Oroya fever is a febrile hemolytic macrocytic anemia of rapid evolution and if untreated of high mortality *B bacilliformis* is readily visible in blood films, becomes progressively more abundant in the blood with the aggravation of the anemia and may infect over 90 per cent of the erythrocytes The mortality rate of untreated Oroya fever is about 40 per cent, if the patient recovers he often develops the second form of the infection verruga peruana This is marked by a generalized eruption of intensely colored red purple vascular papules interspersed with more deep seated nodular elements and is usually benign with a death rate below 5 per cent even when untreated Verruga persists with successive crops for from a month to a year but, unlike Oroya fever, anemia if present is moderate in degree and during all of this period bartonella will not be seen in blood films although it can be cultivated from the blood

Morphology and Biologic Properties of Bartonella

B bacilliformis is a gram negative organism which stains unsatisfactorily with the customary bacteriologic stains but a distinct red violet with Wright s or Giemsa s fluid It is very polymorphous Flagella numbering 1 to 10 and always unipolar have been demonstrated on culture material (Fig 2) but never satisfactorily on the blood forms otherwise the maximum morphologic range is seen in the blood of man where rings commas, disks and rods long short and arranged in chains, may all be seen (Fig 1)

The culture medium most commonly used is Noguchi s leptospira medium a semi solid nutrient agar containing 10 per cent rabbit serum and a small amount of rabbit hemoglobin (0 50% or less) Growth becomes evident in this medium in from a week to 10 days at 28° C sometimes longer in a band about 1 cm wide and about 5 mm below the surface Colonies if minute produce only a faint haze but they may grow to be visible white spherical bodies from 1 to 2 mm in diameter The organisms occur singly and in large and small irregular dense collections with jagged edges 25 to 50 microns or more in diameter Rod forms predominate in young (10 to 20 day) cultures and granules measuring as little as 0 2 × 0 3 micron in older ones Flagella are demonstrable on motile strains by silver impregnation methods and are beautifully shown by the electron microscope after palladium shadowing (Fig 2) The flagella are unipolar arise from the cytoplasm are 1

Diagnosis

Oroya fever usually offers no diagnostic problem once the anemia is pronounced Bartonella can then be found in stained blood films Prior to the anemic period blood cultures can be positive the incentive to make them comes from knowledge of prior residence in an endemic zone and suggestive symptoms of the preanemic period unexplained fever and joint pains A generalized well-developed verruga eruption has a very distinctive appearance Individual elements may resemble those of other eruptive conditions but the histology is usually very different Cultures from the verrugas are usually unsatisfactory due to contamination but cultures of bartonella may be obtained from the blood throughout the course of the eruption The diagnosis of latent infection also depends on blood cultures In all cases cultures if negative should be repeated for particularly during the verruga period it may be very difficult to cultivate bartonella from the blood (Herrer 1953b)

The identification of *B bacilliformis* is based on the following points isolation from patients with a typical case of one of the two forms of the disease or from carriers with a history of residence in endemic areas or from infected sandflies characteristic growth in a semisolid medium pathogenicity for *Macaca mulatta* in which verrugas may be produced the morphology and the cellular situation in vivo and the appearance staining and biochemical reactions in vitro Some strains are nonpathogenic and for these instead of the monkey test tissue cultures can be substituted growth will be both intracytoplasmic and extracellular and within cells the organism will show a tendency to grow in rounded masses

Treatment

Satisfactory clinical results can be obtained even though control of the infection is incomplete If a patient recovers from Oroya fever he may continue to yield positive blood cultures and to develop verrugas but does not die even though still infected presumably because he develops a partial immunity

Oroya fever patients respond dramatically to penicillin (Merino 1945 Aldana and Tisnado 1945) streptomycin (Aldana, Gastelumendi and Dieguez, 1948) chloramphenicol (Payne and Urteaga, 1951) or tetracycline (Araujo 1955) Fever disappears usually in 48 hours or less the bartonella change in morphology the rod forms being replaced by coccuslike types then (after several days if not before) they diminish in numbers whereas the blood count stabilizes and then increases Presumably the antibiotics control the infection and allow the immunologic mechanisms to operate

Choice of antibiotic is regulated by secondary considerations As has been reported for some years secondary salmonella infection of Oroya fever patients is not infrequent and is of poor prognosis Therapeutic agents with activity against both bartonella and the salmonella are often preferred the favorable action of chloramphenicol in such combined infections has been reported (e g Urteaga and Payne 1955) For this reason chloramphenicol is favored over other antibiotics with anti bartonella activity (Cuadra 1956) even though there is some in vitro evidence that it is not the most potent (Wigand, 1952) Symptomatic relief of the anemia is accomplished by transfusions which it may be advantageous to repeat to a total of 3 to 8 liters

Epidemiology

Bartonella is transmitted by one or probably several species of phlebotomus or sand fly indigenous to the endemic regions There are records of a few cases of congenital infection but bartonellosis is not transmitted by ordinary contact The sources of the microorganisms are limited almost exclusively to the two hosts man and phlebotomus whence the importance of the asymptomatic infections The 5 to 10 per cent of the apparently healthy population in endemic areas who have bartonella circulating in the blood as well as postconvalescent carriers and patients serve as possible sources of infection to phlebotomi (Weinman 1944) The fact that repeated cultures were obtained only rarely from infected humans has been cited as evidence against the importance of the human reservoir (Herrer 1953b) This fact may be interpreted equally well as evidence of the inefficacy of the cultural

the clinical entities It is a specific granuloma with three characteristic features It is very vascular as a result of the formation within the element of numerous small caliber blood vessels Proliferating endothelial cells are present in abundance, they frequently occur in masses or islands and lie in edematous connective tissue the whole being infiltrated with a variable number of lymphocytes, plasma cells and polymorphonuclears Lastly bartonella is present in the lesion usually within the cytoplasm of the tissue endothelial cells or in their neighborhood Swollen vascular endothelial cells bulging into the lumen of the vessel which is such a feature of Oroya fever are almost totally lacking (Fig 1-4)

The anemia of Oroya fever is due primarily to blood destruction Erythrocyte loss can be massive and rapid with red blood cell and hemoglobin values reaching 20 per cent of normal values in 96 hours This destruction results from hemolysis (Hurtado *et al* 1938) Transfused tagged erythrocytes obtained either from normal donors or of autologous origin are destroyed at an accelerated rate in Oroya fever patients (Reynafarje and Ramos 1961)

Despite consistent and unequivocal evidence of lysis the intimate mechanism of the hemolysis has resisted analysis No lysin has been obtained from in vivo sources The Coombs test direct and indirect is negative in both phases of the disease (Reynafarjé and Ramos 1961) and bartonella is not hemolytic in vitro For verruga there is at present no information correlating the complex cellular and tissue reactions with the properties or constituents of bartonella

The great variability in response to *B bacilliformis* infection ranging from Oroya fever to verruga not preceded by Oroya and finally to nonclinical latent infection probably is due to variations in both the host and the microorganism Controlled experiments in animals indicate a great range of host susceptibility and of pathogenicity of the organism In Oroya fever both factors probably vary also but since the condition is not reproducible at will in animals the matter is not certain No differences have been detected between bartonella isolated from Oroya fever cases and those obtained from verruga patients They are similar in pathogenicity give various cross immunity tests in animals have the same morphology and behave similarly in vitro Both flagellate and aflagellate strains are pathogenic

Immunity

The fundamental concept is that infection is long continuing and exhibits two successive clinical manifestations of which the second verruga seems to be an actual expression of immunity toward the first form Oroya fever If a patient has undergone Oroya fever, than has had verruga from which he has recovered, usually he will not have a second attack even though exposed But should the second attack occur it will almost invariably be verruga and not Oroya fever This immunity to second attacks does not always signify that the infection has been eradicated On the contrary, bartonella can be cultivated from the blood of recovered patients for long periods of time This latent or asymptomatic infection is a characteristic feature of the bartonelloses animal as well as human Latent infection is not limited to postconvalescence and persons with no history of the disease can be carriers From 5 to 10 per cent of the population in bartonella infected areas are carriers and probably play an important part in maintaining endemicity (Weinman and Pinkerton 1937b) The asymptomatic state may last 15 months at least (Herrer Cornejo *et al* 1959 60)

Complement fixation has been reported with cultures as antigens strains of varying origins gave no significant titer differences in quantitative tests Agglutinins are present during both phases of the disease and in carriers It is doubtful that they play any important part in acquired immunity Limited attempts at immunization with formolized cultures induced agglutinin formation but did not prevent infection Perhaps one or more of the antibodies described above may inhibit the development of bartonella in vitro for Herrer (1953b) found that cultures are more likely to be positive in the first months following infection than later even though infection continues throughout, as was demonstrated by the eventual outcrop of verrugas

Because of the splenic immunity associated with them the group has attracted particular interest *Haemobartonella muris* found in wild and many strains of laboratory white rats provokes a fulminating anemia often with hemoglobinuria and frequently fatal A high percentage of rats are infected with *H muris* yet they show no signs of the disease and react mildly if at all to inoculations of the organism But if the spleen is removed from these rats the microorganisms multiply and the anemia evolves in its acute form The infection is contracted at an early age and usually remains latent probably throughout life unless splenectomy is performed Other procedures may have an effect similar to that of splenectomy i e certain associated infections x ray irradiation α particle irradiation from injected Polonium 210 Quite possibly all of these act to impair the efficiency of the spleen Although haemobartonella infections occur in a great variety of animals none is known in man—the human postsplenectomy iron staining structures Pappenheimer bodies probably not being living organisms (Pappenheimer *et al* 1945) The effect of splenectomy on *B bacilliformis* infections in man is not known but Oroya fever following splenectomy has been described once Eperythrozoon has not been proved to occur in man but has been reported as a possible human infection Splenic control is a prominent feature of eperythrozoon infections The mouse organism *E coccoides* (Fig 1 6) has again attracted attention because it is a striking example of a heterologous latent infection which converts an otherwise benign hepatitis virus infection into a fatal disease at the same time that it induces a many fold increase in virus titer (Niven *et al* 1952)

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method particularly in the presence of antibodies when compared with the efficiency of phlebotomus in favoring growth Meanwhile no important source of bartonella other than man has been discovered (Herrer 1953a)

Once implanted in a region bartonellosis tends to remain almost indefinitely in a focus reported in 1630 the infection has persisted for over 300 years Epidemics arise through infection of a previously nonexposed and therefore nonimmune population Such a population may be introduced within an endemic area to produce an internal epidemic not correlated with geographic extension (e g the 1870 Peruvian outbreak) or there may be an actual invasion of new territory This seems to have been the case in Colombia in 1938 where bartonellosis caused an estimated 4,000 deaths Small outbreaks continue to occur e g in 1959 in the Mantaro valley of Peru where there were an estimated 200 deaths (Herrer and Blancas 1959 60)

Bartonellosis is contracted only in northwestern South America exclusively in mountainous areas solely in the neighborhood of river valleys where the elevation must be neither too high (below 8 000 feet) nor too low (above 2 500 feet) and usually only at night The explanation lies in the biology of the vector The pertinent facts are available for *P verrucarum* in Peru This phlebotomus is restricted to certain areas by requirements of moisture and temperature Above a certain altitude the night temperature is too low below a certain limit the rainfall is insufficient and conditions are too arid Transmission occurs at night becaue the phlebotomi feed then Phlebotomi are the only natural vectors known and *P verrucarum* the only species for which transmission is proved Very probably others are involved In Colombia, where the Peruvian species have not yet been found the epidemic apparently was not caused by the importation of infected sandflies but by infection of native phlebotomi possibly by human carriers the presumptive vector is *P columbianus* (Rozeboom 1947) In the 1959 Mantaro outbreak *P verrucarum* was not recovered *P pescei* is suspect because of distribution coincident with the disease (Herrer and Blancas 1959 60) Experiments involving *Pediculus humanus* have not supported the view that lice may spread the disease (Wigand and Weyer 1953)

Control Measures

DDT appears to have furnished a simple, relatively inexpensive method for community sanitation The application of residue sprays to the inside of residences and sleeping quarters to the outside of doors and windows and to likely breeding spots in the immediate vicinity was shown by Hertig and Fisher (1945) to exert effective phlebotomus control In Peru in an endemic region Corradetti applied these technics in 1949 and succeeded in protecting a group of about 900 men for several months, not only from bartonellosis but to a marked degree from malaria also, and this despite the frequent moving of the work camps as the construction project progressed Thus far resistance to DDT by phlebotomus has posed no problem

Individuals may protect themselves by nightly removal from the phlebotomus zone by the use of insect repellents and DDT as indicated and presumably by the prophylactic use of active antibiotics There is no vaccine of demonstrated efficacy

HAEMOBARTONELLA AND EPERYTHROZOON

The haemobartonella resemble *B bacilliformis* when stained and examined with the light microscope and cause an acute febrile anemia during which they appear in large numbers on the erythrocytes But they do not provoke any condition akin to verruga do not multiply massively if at all within vascular endothelial cells and are widely distributed geographically Their very different response to chemotherapy indicates fundamental metabolic differences in any case they are unsuitable test organisms for anti bartonella activity These differences suggested separation in a distinct genus (Tyzzer and Weinman 1939) a separation reinforced since by the discovery of fundamental structural differences with electron microscope technics (Peters and Wigand 1955)

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35

The Pleuropneumonia and Pleuropneumonialike Organisms

The microorganisms which are designated as pleuropneumonia or pleuropneumonialike organisms are characterized by the lack of a rigid cell wall a very pleomorphic cell morphology and a characteristic colony morphology Except for a few saprophytic strains they require a culture medium enriched with fresh native body protein such as ascitic fluid or blood serum In addition to growing on the surface of a solid medium a portion of the colonies grows into it so that unlike bacterial colonies these cannot be readily rubbed off the surface of the medium The cells are smaller than the usual bacterial cells and stain poorly with the ordinary aniline dyes The colonies are best recognized by staining in situ with a mixture of methylene blue and azure II according to the method of Dienes The organisms are inhibited by the action of specific antibodies in the absence of complement Various generic names which have been proposed in previous systems of classification are Coccobacillus Micromyces Asteromyces Borrelomyces, Bovimyces and Asterococcus The genus name now proposed is *Mycoplasma* and the type species is *Mycoplasma mycoides*

For many years the pleuropneumonia group of microorganisms was composed of the microorganisms which cause pleuropneumonia of cattle and contagious agalactia of sheep and goats Within the last two decades many microorganisms which resemble the organisms of pleuropneumonia in colony morphology and cultural requirements have been isolated under various conditions from man and other species These more recently isolated organisms, for the sake of convenience have been referred to as pleuropneumonialike organisms or, for greater convenience, simply by the abbreviation PPLO Insufficient knowledge of the numerous strains of PPLO is available to permit a precise classification The latest proposals for classification and nomenclature are those of Edward and Freundt (1956), and their recommended terminology is represented by the names which appear in italics in the text

HISTORY

The causative organism of bovine pleuropneumonia was discovered in 1898 by Nocard and co workers North America Western Europe and India are the only areas in the world in which bovine pleuropneumonia does not seem to exist The disease was eradicated from the United States in the middle of the 19th century and introduction of the microorganisms into this country is prohibited by Federal law

Since the small viable elements are capable of passing through bacteria proof filters the organisms were regarded by earlier workers as filterable viruses In cattle the disease involves primarily the respiratory tract—pneu

monia with secondary involvement of the pleura—with an occasional involvement of the joints Joint involvement is a clinical sign which appears with varying degrees of frequency in a majority of the animal species infected with these organisms

In 1925 Bridre and Donatien isolated from sheep and goats the causative organism of contagious agalactia a disease found predominantly in Europe and Algeria The disease involves the joints and the eyes and in lactating sheep and goats the mammary glands Shoetensack in Japan in 1934 isolated similar organisms from dogs with a respiratory type of infection

Since the middle 1930s publications on these microorganisms have appeared more frequently in the scientific literature The rabbit appears to be the only common laboratory animal from which PPLO have not been isolated

The discovery by Klieneberger in 1935 of the dissociation of *Streptobacillus moniliformis* into a small colony variant resembling the pleuropneumonia organisms and designated the L_1 variant initiated a great amount of investigation into the possibility of other bacteria producing similar variants and the possibility of finding the variants in various animals As the first variant was designated L_1 a similar terminology was employed for subsequent isolations This system of naming strains was discontinued after the L_7 term but the L designations still appear occasionally in the scientific literature The L terms had the following original connotations

L_1—the small colony variant produced by strains of *S moniliformis* and described by Klieneberger in 1935 Edward stated in 1950 that while the L_1 organisms have the general characteristics of PPLO they are quite distinctive in their cellular morphology and they frequently produce growth which resembles that of ordinary bacteria

L_2—the PPLO discovered by Klieneberger in 1935 to be associated with a small streptococcus or streptobacillus isolated from the nasopharynx of guinea pigs

L_3—the PPLO isolated by Klieneberger and Steabben in 1937 from the lungs of rats exhibiting chronic bronchopneumonia or bronchiectasis A similar strain was isolated from the brain of an apparently healthy mouse by Findlay *et al* in 1939 The name *Mycoplasma pulmonis* has been proposed for this strain

L_4—a term employed for the only strains of PPLO believed to possess pyogenic properties These organisms were isolated from rats by Klieneberger in 1939 and by Woglom and Warren in 1939 Arthritis is invariably produced when these organisms are injected into the foot pads of rats and mice The organisms are similar serologically to the L_7 organisms but they are more pathogenic for mice by intracerebral inoculation than the L_7 The name *Mycoplasma arthritidis* has been proposed

L_5—a term employed for the only strains of PPLO which produce rolling disease in mice The organisms were encountered by Findlay *et al* in 1938 during the brain to brain passage in mice of neurotropic yellow fever virus and the virus of lymphocytic choriomeningitis The intracerebral injection of pure cultures of the organisms did not produce rolling disease but the culture mixed with brain suspension containing any one of several viruses did produce the characteristic symptoms Mice and the field vole *Microtus agrestis* are the only susceptible animals The name *Mycoplasma neurolyticum* has been proposed

L_6—strain of PPLO isolated by Findlay *et al* in 1939 from the brains of mice The organisms are related to L_5 organisms but they differ from these in growth and serologic characteristics In morphologic and staining properties the organisms can be mistaken for *Eperythrozoon coccoides*

L_7—strains of PPLO isolated from arthritic joints of rats by Findlay *et al* in 1939 It was shown in 1940 by Klieneberger that the organisms are identical with the L_4 organisms

It is current practice to designate as L forms those pleuropneumonialike organisms which can revert to a bacterial form and as PPLO those for which no reversion has yet been demonstrated

The first known encounter with PPLO in man is perhaps the work of Gey (personal communication) who succeeded about 1935 in isolating several dozen lines from human placental cord blood which was being employed in tissue culture work However the

identity of these agents as PPLO was not possible until the special technics for working with these organisms were developed by Dienes Currently the PPLO are a problem in tissue culture work as many lines of cell cultures are contaminated with these organisms Primary tissue cell cultures may be contaminated with PPLO the rate of contamination may be in the range of 80 to 90 per cent in laboratories employing antibiotics in the tissue culture media (Barile *et al* 1962) Some of the strains of PPLO contaminating tissue cell cultures appear to be antigenically similar to serologic types 1 and 2 of *Mycoplasma hominis* which were originally isolated from the human genito urinary tract (Bailey *et al* 1961)

CULTIVATION AND BIOLOGIC PROPERTIES

PPLO are the smallest microorganisms which have been cultivated on cell free media Their nutritional requirements except for the saprophytic strains are different from those of all other microorganisms It is essential to start with a rich basal medium and to supply the additional growth factors

In most cases an infusion from beef heart muscle is employed as infusions from other tissues are less suitable To the infusion are added 0 5 per cent NaCl and appropriate peptone to the concentration of 1 per cent The brand of peptone is important as not all brands support growth of PPLO equally well Bacto peptone was found to be the best of the available American peptones (Morton *et al* 1951) For solid medium 1 4 per cent agar may be added The organisms are so sensitive that they may not grow in the presence of certain lots of bacteriologic agar (Lynn and Morton 1956) It is desirable to have the medium as soft as practicable since the colonies grow into the medium

The organisms require an alkaline environment for optimum growth so the final reaction of the medium should be about pH 7 6 to 7 8 The basal medium requires enrichment with a native body protein such as human ascitic fluid or blood serum Ascitic fluid in the final concentration of 20 to 30 per cent appears to be the best enrichment but often this is impractical Blood serum in a final concentration of 10 to 20 per cent is frequently used rabbit horse or beef serum is usually used but the serum from no one species is uniformly satisfactory

Often the sera of some species or certain lots of serum from the same species either do not promote growth or inhibit the growth of some strains This lack of a growth promoting property in certain sera may be due to the presence of specific antibodies Although beef serum is unsatisfactory as an enrichment for growing many human strains of PPLO a way was found for separating the growth factor or factors from the toxic fraction (Smith and Morton 1951) The complete basal medium in the dehydrated form and a serum fraction containing the growth factor in a concentrated form are available commercially so that cultivation of PPLO has become as practicable as cultivating many species of bacteria

The growth factor has been shown to be a lipoprotein containing only bound cholesterol and phospholipid (Smith Lecce and Lynn 1954) and is not known to be required by any other microorganisms It is very similar to alpha 1 lipoprotein and can be replaced with lipid free protein which does not appear to be specific and cholesteryl laurate and lecithin of animal origin The lipid requirement for growth was also demonstrated by Edward and Fitzgerald (1951) Only the amino acids alanine arginine glutamic acid glycine leucine lysine tryptophane and valine were found in the purified growth factor (Smith and Morton 1952) The purified form of the growth factor is nonantigenic in rabbits (Smith Morton and Keller 1953) While the lipoprotein factor allows the growth of the majority of strains of PPLO there are strains which require other substances For example Edward and Fitzgerald (1952) found deoxyribonucleic acid to be a growth factor for certain strains when first isolated from cattle The growth of some strains is dependent on or is enhanced by the presence of yeast extract in the culture medium and some strains may even require serum from a particular species of animal *Mycoplasma pneumoniae* requires yeast extract (freshly prepared) and fresh horse serum (Chanock Hayflick and Barile 1962) Different lots of serum from the same species are not always equally satisfactory (Baernstein and Quilligan 1963)

The function of the serum appears to be to provide sterol in a utilizable form for those strains which are unable to synthesize it for incorporation into the nonsaponifiable lipids in the cell membranes The non sterol re quiring strains are able to synthesize the nonsaponifiable lipids *de novo* but they are capable also of adsorbing sterols

The growth of PPLO can be measured quantitatively by some of the methods com monly employed for estimating numbers of viable bacteria e g turbidimetric measure ment of concentrated cell suspensions cellu lar nitrogen and counts of surface colonies resulting from the deposition of measured amounts of cell suspension on the surface of appropriate solid medium (Smith 1956) Usually no noticeable turbidity is produced by the growth of PPLO in liquid media The amount of cellular nitrogen in broth cultures of PPLO is about 0 01 that of bacterial cul tures

In general the organisms can be culti vated under aerobic conditions Some oral strains and an occasional genital strain from man require anaerobic cultivation The pres ence of 10 per cent CO in the environment offers no advantages if the culture medium is satisfactory and it may be detrimental if the medium is slightly unsatisfactory Growth of the parasitic strains takes place at 37 C The organisms appear to reproduce by bi nary fission with a generation time calcu lated to be 3 27 ± 0 78 hours Most of the strains of human origin grow in the develop ing chick embryo without producing notice able pathology or death of the embryo Sul livan and Dienes in 1939 encountered one strain of PPLO which grew only in embryos that had been killed by chilling and Swift in 1941 observed that in general PPLO grew better on dead sterile chorio-allantoic mem branes than on living membranes Many of the strains from other species of animals are pathogenic for the embryos The organisms grow readily in tissue cultures and currently are an important factor as contaminants in tissue cultures employed for propagating viruses The PPLO cells may lead an intra cellular existence since they are found within the tissue cells as well as extracellularly (Edwards and Fogh 1960)

Because of the frequent occurrence of PPLO along with bacteria in clinical ma terials the frequency with which cultures of PPLO become contaminated with bacteria the small colonies produced by PPLO their slow rate of growth and their requirement for a rich medium it is often necessary to suppress the growth of bacteria while per mitting PPLO to grow The organisms differ from gram positive bacteria in not being inhibited by crystal violet and from gram negative bacteria in not being inhibited by potassium tellurite Thallium acetate first employed by Edward in 1947 appears to possess advantages over a combination of selective bacteriostatic agents (Morton and Lecce 1953) Penicillin may be employed if the bacteria to be inhibited are penicillin sensitive but its use poses the possibility of converting bacteria into L forms

Resting cells of PPLO are not very resist ant to physical factors as compared with vegetative cells in general A typical strain of PPLO had a half life of 15 min 45 min 35 min 20 min and over 24 hours at 37 C when suspended respectively in distilled water 0 85 per cent NaCl solution M/15 phosphate buffer pH 7 0 Ringer s solution and the culture supernate Survival was found to be only slightly better at room tem perature At 4 C the half life periods were more than 6 days in each case (Smith and Sasaki 1958) At 50° C the half life was 2 min or less no organisms survived 7 to 10 minutes exposure at this temperature PPLO are much more susceptible than most bac teria to the deleterious action of agents which alter surface tension as evidenced by their susceptibility to the action of soaps and to the action of bile acids In 3 bile acids tested—cholic desoxycholic and lithocholic—a direct relationship was noted between the destruction of PPLO and the degree of surface tension depression due to the number of polar groups (Smith and Sa saki) Surprisingly PPLO were found to be relatively insensitive to osmotic shock This may be because of their pliable limiting cell membrane in place of the rigid cell wall found in bacteria In regard to their resist ance to phenol hydrogen peroxide and ultra violet light members of this group resemble bacterial vegetative cells (Warren 1942)

Metabolically PPLO are a heterogenous group Many strains are inert toward carbo hydrates as shown by the usual fermentation

identity of these agents as PPLO was not possible until the special technics for working with these organisms were developed by Dienes Currently the PPLO are a problem in tissue culture work as many lines of cell cultures are contaminated with these organisms Primary tissue cell cultures may be contaminated with PPLO the rate of contamination may be in the range of 80 to 90 per cent in laboratories employing antibiotics in the tissue culture media (Barile *et al* 1962) Some of the strains of PPLO contaminating tissue cell cultures appear to be antigenically similar to serologic types 1 and 2 of *Mycoplasma hominis* which were originally isolated from the human genitourinary tract (Bailey *et al*, 1961)

CULTIVATION AND BIOLOGIC PROPERTIES

PPLO are the smallest microorganisms which have been cultivated on cell free media Their nutritional requirements except for the saprophytic strains are different from those of all other microorganisms It is essential to start with a rich basal medium and to supply the additional growth factors

In most cases an infusion from beef heart muscle is employed as infusions from other tissues are less suitable To the infusion are added 0 5 per cent NaCl and appropriate peptone to the concentration of 1 per cent The brand of peptone is important as not all brands support growth of PPLO equally well Bacto peptone was found to be the best of the available American peptones (Morton *et al*, 1951) For solid medium 1 4 per cent agar may be added The organisms are so sensitive that they may not grow in the presence of certain lots of bacteriologic agar (Lynn and Morton 1956) It is desirable to have the medium as soft as practicable since the colonies grow into the medium

The organisms require an alkaline environment for optimum growth so the final reaction of the medium should be about pH 7 6 to 7 8 The basal medium requires enrichment with a native body protein such as human ascitic fluid or blood serum Ascitic fluid in the final concentration of 20 to 30 per cent appears to be the best enrichment but often this is impractical Blood serum in a final concentration of 10 to 20 per cent is frequently used rabbit horse or beef serum is usually used but the serum from no one species is uniformly satisfactory

Often the sera of some species or certain lots of serum from the same species either do not promote growth or inhibit the growth of some strains This lack of a growth promoting property in certain sera may be due to the presence of specific antibodies Although beef serum is unsatisfactory as an enrichment for growing many human strains of PPLO a way was found for separating the growth factor or factors from the toxic fraction (Smith and Morton 1951) The complete basal medium in the dehydrated form and a serum fraction containing the growth factor in a concentrated form are available commercially so that cultivation of PPLO has become as practicable as cultivating many species of bacteria

The growth factor has been shown to be a lipoprotein containing only bound cholesterol and phospholipid (Smith Lecce and Lynn 1954) and is not known to be required by any other microorganisms It is very similar to alpha 1 lipoprotein and can be replaced with lipid free protein which does not appear to be specific and cholesteryl laurate and lecithin of animal origin The lipid requirement for growth was also demonstrated by Edward and Fitzgerald (1951) Only the amino acids alanine arginine glutamic acid glycine leucine lysine tryptophane and valine were found in the purified growth factor (Smith and Morton 1952) The purified form of the growth factor is nonantigenic in rabbits (Smith Morton and Keller 1953) While the lipoprotein factor allows the growth of the majority of strains of PPLO there are strains which require other substances For example Edward and Fitzgerald (1952) found desoxyribonucleic acid to be a growth factor for certain strains when first isolated from cattle The growth of some strains is dependent on or is enhanced by the presence of yeast extract in the culture medium, and some strains may even require serum from a particular species of animal *Mycoplasma pneumoniae* requires yeast extract (freshly prepared) and fresh horse serum (Chanock Hayflick and Barile 1962) Different lots of serum from the same species are not always equally satisfactory (Baernstein and Quilligan 1963)

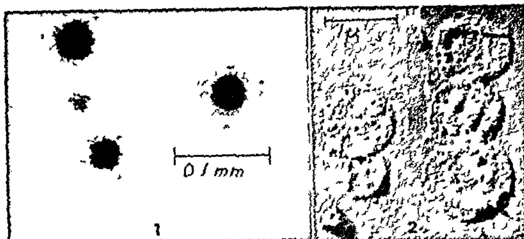


Fig 1 (*Left*) Photomicrographs of colonies of pleuropneumonialike organisms stained by the method of Dienes The colonies on the left are from strain No 60 which was isolated from the human cervix For the most part th colonie showed the typical fried egg appearance but an occasional small, dense colony which measured about 25 μ was present such as the colony between the two lar e colonies The colony on the right is from strain No 48 which was isola d as a pure culture from the male urethra (Fig 1 Leberman *et al* J Urol 6 167)

Fig 2 (*Right*) Electronmicrographs of chromium shadowed cells of Campo strain of pleuropneumonialike organisms from man (*Mycoplasma hominis* serologic type 2) from a 48 hour culture on solid medium Note the holes in the cell membranes and the flatness of the cells which give the appearance that the contents of the cells have escaped possibly during the drying of the specimen for electronmicroscopy (Figs 13 and 15 Morton *et al* J Bact 68 697)

In some cases the organisms produce suspensions which are unsuitable for agglutination tests The complement fixation reaction is a practical serologic method but suspensions of PPLO are frequently anticomplementary The PPLO cells can be absorbed to latex (0 81 micron diam) and used as antigen in an agglutination test (Morton 1962) Significant titers have been reported for sera from patients with deeply localized infections with PPLO (Melen and Gotthardson 1955 Stokes 1955) As in the case of other infectious diseases animals that have recovered from infections may be immune to subs quent infections frequently without exhibiting specific antibodies in their sera (Edward 1954)

An interesting property of organisms of this group is that their growth is inhibited by specific antibodi s in the absence of complement This characteristic is analogous to the neutralization test with viruses The growth of the L type variant of *Proteus vulgaris* was inhibited by its specific antibody but not by antibodies to the bacillary forms of *P vulgaris* This indicates important differences between the L and the bacillary forms of the same organism

The hemagglutination and hemagglutination inhibition tests are used extensively with the avian strains of PPLO but the human strains do not exhibit these properties

Interesting from the standpoint of the ecology of PPLO associated with man is the finding by Freundt (1954) that 2 strains were identical serologically with the organism of bovine pleuropneumonia and the finding by Smith Peoples and Morton (1957) that a poultry strain and a human strain cross reacted serologically Strains of PPLO isolated from goats swine and a cat have been obs rved to cross react serologically with one of the serologic types of PPLO originally isolated from the human genitouri

tests Other strains from man and other species are capable of utilizing the test carbohydrates and do so with the accompanying production of acid and no gas (Edward 1954 Freundt 1954) Of 207 human strains of PPLO 189 were found to be biochemically inert in fermentation tests 2 strains had fermentation reactions identical with the organism of bovine pleuropneumonia and the balance of the strains had variable fermentation reactions (Freundt 1954) The organisms have a very active monohydric alcohol dehydrogenase less dehydrogenase activity with lactate fructose and ribose and no activity with glucose Dehydrogenase activity is also evident from the ability of the organisms to reduce methylene blue to a varying degree and to reduce tetrazolium compounds (Somerson and Morton 1953) In a synthetic medium the organisms appear to derive their entire energy sources from amino acids and Sn^{++} serves an essential role in some manner Attempts to demonstrate transaminases have not been successful Utilization of arginine glutamine glutamic acid aspartic acid tryptophane and tyrosine and under aerobic conditions only histidine leucine and threonine has been demonstrated Among the limited biochemical activities of these organisms deamidation appears to be a significant function That of glutamine appears to be brought about by two distinct reactions One reaction is a simple hydrolytic deamidation and the other is a reaction which requires Mg^{++} phosphate and adenosinediphosphate Glutamic acid is converted to a cyclic compound which is then reduced to proline through the mediation of reduced triphosphopyridine nucleotide (Smith 1957a) Arginine is converted to citrulline and this is converted to ornithine which is also utilized (Smith, 1957b) The break down of glutamine and citrulline gives rise to the formation of adenosinetriphosphate

PPLO and PPLO infected tissue cultures degrade arginine whereas PPLO free tissue cultures do not (Schimke and Barile 1963) Therefore arginine deaminase activity may be used as a means of detecting PPLO contamination of tissue cell cultures

None of the strains of PPLO isolated from man has been observed to produce indole H S or oxidase or to reduce nitrates to nitrites *M pneumoniae* produces hemolysis of the β type when tested with guinea pig erythrocytes but not with human horse or rabbit erythrocytes Other strains of PPLO from man either are inert toward erythrocytes or produce hemolysis of the α type (Clyde 1963 Somerson *et al* 1963)

Colony morphology differs considerably from that of bacteria and varies less among strains within the group It is one of the properties which is detected most easily and is looked for early when attempting to detect these organisms The diameters of the colonies vary from approximately 10 microns to 1 mm but usually are about 250 to 750 microns Well isolated colonies are usually circular with an entire edge although Shepard (1956) reported tiny colonies (T forms) 10 to 12 microns in diameter which were irregular in outline Because of their small size the colonies are examined best with the low power of the microscope (100×) and transmitted light The colonies usually grow into the medium so they are not rubbed off when an inoculating loop is drawn over the surface of the culture Well isolated colonies developing under optimal conditions usually show the 'fried egg appearance when viewed by transmitted light This appearance is caused by the fact that the central portion of the colony grows into the medium and the surrounding portion grows on the surface The appearance is shown in photographs published by Edward in 1950 and by Somerson and Morton (1953)

The surface of the colonies is raised and varies in texture from a slight pitting to a coarse lacy appearance as shown in photographs published in 1950 by Nelson and by Edward In addition there is sometimes a centrally located small peak (Sabin, 1941) or depression (Edward) The colonies may be further identified by staining in situ by the method of Dienes (Fig 1)

Individual organisms are not stained readily by the dyes usually employed to stain bacteria and are gram negative Giemsa s or Wayson s stain is usually employed

Serologically the numerous organisms in this large group comprise many antigenic types Klieneberger (1940) found distinct serologic types by the agglutination reaction

nary tract (Morton unpublished data) A serologic relationship between the human type 1 PPLO of urogenital origin and human oral strains also has been demonstrated (Taylor Robinson *et al* 1963)

The knowledge of the morphology of PPLO is not as precise as that pertaining to bacteria partly because of the small size of the cells the poor staining with ordinary dyes and the fact that the colonies of PPLO are quite distinct Phase-contrast microscopy and electron microscopy have been helpful Freundt gave a good description in 1952 of the growth of the bovine pleuropneumonia organisms Both granules and filaments were demonstrated The smallest elements which are capable of germination and are filterable are the so called elementary corpuscles The formation of branching mycelia is also part of the morphology of these organisms and is a characteristic which differentiates them from bacteria Growth has been observed to take place by the extrusion of thin filaments from the elementary corpuscles These filaments are without septa and may reach a length of 150 microns As the organisms become older these homogenous filaments become transformed into long chains of regularly spaced closely set highly refractive uniformly shaped small bodies Regularly spaced constrictions take place in the walls of the filaments between the small bodies until finally the small bodies (elementary corpuscles) are held together by a delicate filament which is devoid of protoplasm The elementary corpuscles are released when the constrictions completely sever the cell wall

In a study of 207 human strains of PPLO Freundt (1954) found two which were identical with the organisms of bovine pleuropneumonia in their biochemical reactions and in their cellular morphology as revealed by the electron microscope The majority of the strains (189) were characterized by a difference in morphology and a lack of biochemical activity The initial mycelial forms of the organisms in this large group were rather stout short and stiff The fine delicate filaments so characteristic of the pleuropneumonia organisms were observed only rarely With these organisms the filaments tended to fragment into cocci and irregularly shaped rods at an early stage of growth The balance of the strains about 7 per cent were intermediate between these two groups in their morphology Somewhat similar morphologic forms appear in electron micrographs of organisms causing agalactia and pleuropneumonia in goats (Klieneberger Nobel and Cuckow 1955) Electron micrographs of human strains and of a poultry strain showed spherical bodies of less than 1 micron some of which were undergoing division (Morton *et al* 1954) (Fig 2) Flagella never have been observed on cells of PPLO

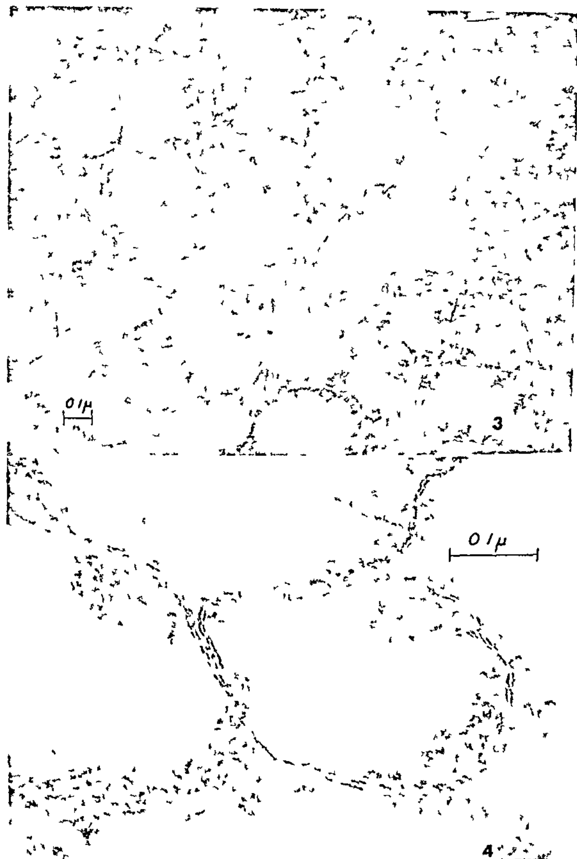
More recent studies with the electron microscope by Van Iterson and Ruys (1960) indicate that colonies produced by human strains of PPLO are made up for the most part of vesicles with simple structure such as shown in Figure 3 A much higher magnification of these basic structures shows the double dark layered limiting membrane of about 75 Å in width

RELATIONSHIP OF PLEUROPNEUMONIA AND PLEUROPNEUMONIA LIKE ORGANISMS TO BACTERIA

The pleuropneumonia and pleuropneumonialike organisms appear to be between the viruses and the rickettsiae on the one hand and the bacteria on the other The pleuropneumonia organisms form a rather homogeneous group and they are distinct in their general properties from the viruses the

FIG 3 (*Top*) Electronmicrograph of an ultra thin section of a colony of pleuropneumonialike organisms from man (*Mycoplasma fermentans* strain V58 serologic type 3) The tightly packed units show internal structures of dense areas and fine fibrils (Fig 10 Van Iterson and Ruys J Ultrastruct Res *3* 382)

FIG 4 (*Bottom*) Electronmicrograph of an ultra thin section of a colony of pleuropneumonialike organisms from man (*Mycoplasma fermentans* strain V26) at very high magnification (approx × 213 500) The limiting membrane about 75 Å thick consists of 2 dense outer layers about 20 Å thick and a lighter inner zone about 35 Å thick (Fig 7 Van Iterson and Ruys J Ultrastruct Res *3* 282)



FIGURES 3 and 4 (*Caption on facing page*)

of certain microorganisms to disease may come about if it is established that some of the PPLO isolated from the body are related to bacteria especially to pathogenic bacteria Since the L_1 variant of *S moniliformis* has been isolated from rats in the absence of *S moniliformis* there may be comparable circumstances in the case of other bacteria and man The Campo strain of PPLO which was isolated from the human urethra has been maintained on artificial medium for over 15 years During certain periods liquid cultures of this strain as well as those of another strain from the human urethra have been observed repeatedly to contain coryne bacteria (Smith *et al* 1957) Statistically it appears very unlikely that the corynebacteria are contaminants and moreover the PPLO and the associated corynebacteria are related serologically and have similar fermentation reactions Wittler *et al* (1956) obtained corynebacteria from a urethral strain of PPLO by cultivating the latter in tissue cultures These strains of corynebacteria were identical serologically and biochemically with a strain of corynebacterium originally isolated from the same urethral exudate at the time the PPLO were isolated and were related antigenically to that strain of PPLO Wittler *et al* suggested that with further study other human genital strains of PPLO may be found to be L forms of *Corynebacterium* spp Amies and Jones (1957) present a similar view in regard to the relationship of certain PPLO from the genitourinary tract to *Haemophilus vaginalis* In the field of veterinary medicine a comparable view had been expressed by McKay and Taylor (1954) when these workers recovered from PPLO associated with chronic respiratory disease of chickens and sinusitis of turkeys a gram negative rod which resembled *Haemophilus gallinarum* They suggested that the agent of these infections was the L form of a true bacterium and not a PPLO

DISTRIBUTION IN NATURE EPIDEMIOLOGY AND RANGE OF PATHOGENICITY

It is now apparent from studies during the past few years that PPLO are not only very prevalent in man during health and disease but are prevalent in many species of animals with which man has contact The finding that some of the strains of PPLO isolated from man react serologically with strains isolated from other species of animals indicates that more needs to be known about the ecology of these microorganisms in order to understand better their role in disease

MAN

The first isolation of PPLO from a pathologic process in man was from the genitourinary tract by Dienes and Edsall in 1937 and the greatest amount of work in relation to the human strains of PPLO has pertained to the study of their prevalence nature and possible relationship to pathology in that region The organisms are more likely to be present in the genitourinary tract if some pathologic condition is present This association was found by Melen and Odeblad (1951) to be statistically significant In culturing the urethra the vagina and the cervix of 58 gynecologically normal women ranging in age from 16 to 44 years these investigators in 1952 failed to find the organisms in 13 virgins (mean age 21 years) although Freundt found the organisms in cultures from the vulva of 3 of 50 girls under 3 years of age and in the vaginal cultures from 4 of 6 adult virgins If a single area or combination of any of the three areas—the urethra, the cervix or the vagina—is positive for PPLO it invariably is the vaginal culture in which the PPLO appear Therefore cultures from the vagina will give a good indication if PPLO are present in the lower female genital tract The fact that PPLO grow better in an alkaline environment may be an explanation for their scarcity in the healthy vagina Freundt found a distinct relationship between the pH of the vagina the vaginal flora and the presence of PPLO The PPLO were usually absent in the vaginas with the lower range in pH (3 7 to 5 0) and the flora exclusively Doderlein s bacillus A similar relationship between the presence of PPLO and lactobacilli in the vagina was noted by Huijsmans Evers and Ruys (1956) Morton *et al* (1952) failed to culture the organisms from the cervix of 12 healthy married women, while Melen and Odeblad in the same year found the organisms in 5 of 32

rickettsiae or the bacteria The pleuropneumonialike organisms form a heterogeneous group Some strains resemble the pleuropneumonia organisms in their morphology and in their serologic and biochemical reactions while other strains have more of the properties of bacteria in their nutritional requirements and cellular morphology

Klieneberger s discovery in the mid 1930 s of the small colony variant (the L_1 variant) of *S moniliformis* which resembles the colonies of the pleuropneumonia organisms stimulated efforts to determine whether this new type of bacteria existed free in nature and whether similar forms could be produced from other bacteria Impetus was also added to the work by the discovery that the L_1 variant is extremely resistant to penicillin Not only can penicillin be used to separate the penicillin resistant L forms from the more penicillin sensitive bacterial forms when both are present in mixed cultures but penicillin stimulates the production of L forms from bacteria In their review Dienes and Weinberger (1951) listed 13 genera of bacteria from which L forms had been produced *Bacillus Bacteroides Clostridium Escherichia Flavobacterium Hemophilus Neisseria Pasteurella Proteus Salmonella Shigella Streptobacillus* and *Vibrio* To this list can now be added *Azotobacter* (Eisenstark *et al* 1950) *Brucella* (Nelson and Pickett 1951) *Corynebacterium* and *Staphylococcus* (Dienes and Sharp 1956) *Streptococcus Diplococcus* (Madoff and Dienes 1958) *Listeria* (Suchanova and Patocka 1957) *Pseudomonas* and *Sarcina* (Ward and Martin 1962) and *Serratia* (Bandur and Dienes 1963)

For practical purposes the microorganisms resembling the pleuropneumonia organisms which are isolated from various sources without any apparent relationship to a known bacterium are designated PPLO When similar forms are isolated from a bacterial culture and bear some relationship to the culture the forms are referred to as L forms and their colonies as L-type colonies ' The demonstration of the characteristic colonies becomes the important criterion in determining whether one is dealing with L forms rather than with the pleomorphic large swollen forms of bacteria The development of L-type colonies is interpreted by Dienes and Sharp (1956) as evidence that viable granules in ' L forms have germinated A definite relationship has been demonstrated for the L_1 variant and its parent culture of *S moniliformis* and between certain L forms and their parent *Proteus* cultures (Dienes Weinberger and Madoff, 1950) In the case of one strain of *Streptococcus pyogenes* the L form continued to produce the type specific protein (type 19) In two strains studied the L forms failed to produce the group-specific polysaccharide (Sharp *et al* 1957) and in 3 strains of group D streptococci the L forms were devoid of their group D antigen and cell wall polysaccharide When one of the L strains reverted to the coccal form the two antigens were present again (Hijmans 1962)

The L forms isolated from various strains of bacteria differ considerably and the L forms isolated from the same strain of bacterium may differ L forms frequently are more difficult to cultivate in liquid media than are PPLO (Dienes 1953) and this property is sometimes employed as a means of differentiating the two types of organisms However Medill and O Kane (1954) grew the L forms of *Proteus* in a synthetic liquid medium and suggested that a reason for poor growth in natural media might be the presence of an inhibitor or inhibitors in some of the natural products Serum or plasma might function as a detoxifying agent rather than as a supplier of necessary nutrients Nevertheless Edward pointed out in 1953 that there may be differences in the nutritional requirements of L forms and PPLO

The L forms may arise spontaneously in bacterial cultures or they may be produced when actively metabolizing bacterial cells are subjected to some unfavorable condition The substances which have proved to be useful in producing L forms are penicillin and a few other antibiotics glycine and a few other amino acids (Dienes and Zamecnik 1952) antibodies in the presence of complement bacteriophage and such unfavorable conditions for normal bacterial growth as are brought about by high concentrations of electrolytes (Dienes and Sharp 1956) refrigeration bacterial antagonism and tap water

A better understanding of the relationship

phimosis ulcerative balanitis with inflammatory phimosis and erosive balanitis by Ruiter and Wentholt (1952) cystitis (Berg *et al* 1957) prostatitis and epididymitis

The strains of PPLO isolated from the genitourinary tract of man are diverse in their antigenic structure To one group of strains Edward and Freundt (1956) have given the name *Mycoplasma hominis* types 1 and 2 The majority of the strains which were isolated in England as well as some strains which had been isolated in France and the United States could be classified provisionally as type 1 Several strains all of which had been isolated in the United States differed from the type 1 strains and were classified provisionally as type 2 The strains of PPLO isolated by Ruiter and Wentholt from cases of balanitis and fusospirillary vulvovaginitis which differed culturally and serologically from the usual human genital strains and were designated G strains were classified as human type 3 strains and called *Mycoplasma fermentans* Genitourinary strains are encountered which cannot be classified into the above types (Huijsmans Evers and Ruys 1956)

Venereal transmission must be considered as a possibility when the organisms are present in either the male or the female genitourinary tract Dienes and Smith in 1942 reported two instances where there was the possibility of transmission of PPLO between husband and wife during intercourse in a third case a young girl developed an acute vaginitis 4 days after intercourse and an abundant growth of PPLO was obtained from the discharge Two additional instances were reported by Morton *et al* in 1951 in which similar PPLO were isolated from both the husband and the wife The same type of PPLO were isolated by Ruiter and Wentholt from a woman with severe leukorrhea and from the prostatic fluid from her husband and Melen and Linnros obtained a profuse growth of PPLO from urethral vaginal and cervical cultures from the wife of a man with nongonococcal urethritis Schaffarzick and Mankle (1953) have reported a condition where it appeared that the male harbored an infection with PPLO transmitted the infection to a female contact and later was reinfected by her With venereal diseases in general both sexual partners must be taken into consideration and it now appears that in the case of suspected infections of the genitourinary tract due to PPLO both partners must be examined If PPLO are present in both both should be treated Transmission may be by contact other than venereal since a technician working with urethral and conjunctival specimens containing PPLO developed conjunctivitis and urethritis from which PPLO were isolated (Kuzell and Mankle 1960)

PPLO were first cultivated from the oral region in 1951 when Morton *et al* detected the organisms in the saliva of 46 of 100 individuals and Smith and Morton (1951) detected them in the throat cultures of 38 of 114 individuals Freundt (1953) isolated them from throat washings of 43 per cent of 95 women Although the association of the presence of PPLO in the oral region and the presence of the organisms in an infectious process may be coincidental two reports are of interest Paine *et al* (1950) isolated an alpha streptococcus and a PPLO from a brain abscess in an individual who had the stem of a smoking pipe thrust into the brain during an altercation and Carlson *et al* (1951) cultured PPLO from the blood of a child who gave a history of a human bite PPLO were isolated in pure culture postoperatively from a bronchopleural fistula after the removal of a drainage tube and later from fluid in the pleural cavity from a patient with bronchiectasis (Stokes 1955) The oral strains of PPLO differ from strains isolated from the genital tract in agglutination reactions colony morphology and nutritional requirements (Dienes and Madoff 1953 Nicol and Edward 1953 Shklair *et al* 1962) The name *Mycoplasma salivarium* has been proposed for the strains of PPLO which have been isolated from the oral region in man

The name *Mycoplasma pneumoniae* has been proposed for the PPLO which causes some cases of primary atypical pneumonia (Chanock *et al* 1963) This agent was formerly known as the Eaton agent or the virus of primary atypical pneumonia and is discussed in the chapter on atypical pneumonia *M pneumoniae* differs from the oral and the genital strains of PPLO in produc

nonpregnant married women and in 3 of 13 pregnant women Klieneberger Nobel (1945) cultured the organisms only twice and then there was only sparse growth from 45 normal pregnant women in contrast with finding the organisms in 40 per cent of 45 women attending a venereal disease clinic

The association of PPLO and *Neisseria gonorrhoeae* in cervical cultures was found by Somerson *et al* (1955) to be statistically significant and no such relationship was found between the presence of PPLO and leukorrhea gross genital pathology phase of menstrual cycle or previous penicillin therapy in the group of 86 women studied The possible association of the presence of PPLO and previous penicillin therapy is of interest because of the ability of penicillin to produce the L forms of many bacteria and to reduce the number of penicillin sensitive organisms in the normal flora Schaub and Guilbeau (1949) isolated PPLO from the postpartum uterus of 3 per cent of the untreated patients and from 20 per cent of the patients who had received penicillin In women who had re ceived penicillin 70 per cent of the cervical cultures yielded PPLO while 26 per cent of 300 consecutive gynecologic patients and 17 per cent of the women with noninflammatory diseases were positive for PPLO (Randall *et al* 1950)

Various other pathologic conditions in the female genitourinary tract from which PPLO have been isolated are chronic vulvitis (Homma and Kusano 1954) fusospirillary vulvovaginitis vaginitis (Bercovici *et al* 1962) cervicitis cystitis (Dienes Ropes Smith, Madoff and Bauer 1948) inflamed uterine tube and salpinx and ovarian abscesses (Gotthardson and Melén 1953)

The finding in 1946 by Salaman *et al* of PPLO in urethral discharges from cases of nongonococcal urethritis stimulated investi gations into the possible etiologic role of these organisms in this condition the inci dence of which now exceeds that of gonorrhea The etiology of nongonococcal ureth ritis remains obscure but it appears that more than one agent may be responsible for it The situation is further complicated by the fact that frequently PPLO are isolated from the normal apparently healthy, male urethra Morton *et al* (1952) failed to iso late PPLO from the semen of 28 healthy individuals who were being studied in a fertility research laboratory but Freundt (1956a) in Denmark isolated PPLO from the urethra of 15 (53 6%) of 28 healthy (trivial skin complaints but healthy as re gards their genitals) individuals compared with an incidence of 30 3 per cent from cases of nongonococcal urethritis 50 3 per cent from cases of subclinical nongonococcal urethritis, 34 per cent from cases of acute gonorrhea, and 47 8 per cent from cases of epididymitis without urethritis The studies of Shepard introduce another facet to the already complex situation He found PPLO in the urethral cultures of 33 per cent of 57 normal Negro male medical students and in only 2 per cent of 55 normal white male medical students and staff members in North Carolina The student who yielded the posi tive culture was one of 24 married individu als in the group Of the 38 Negro men with nongonococcal urethritis PPLO were re covered from 53 per cent, and the organisms were recovered from 56 per cent of the ure thritis free Negro men visiting a venereal dis ease clinic Melen and Linnros (1952) found the incidence of PPLO in nongonococcal urethritis and healthy individuals to be only 18 per cent and 16 per cent respectively Against these data one has to weigh the evi dence that in many instances PPLO are the only organisms or the predominating organ ism cultivated from a pathologic process and when the organisms disappear during the course of therapy there is also a re mission of symptoms Three patients with urethritis were treated successfully with streptomycin and 7 patients were treated success fully with oxytetracycline, 2 of 3 patients responded to streptomycin therapy and 1 patient with urethritis of long duration was treated successfully with streptomycin (Melen and Linnros) Recovery following treatment with chlortetracycline was more frequent in those patients in whom PPLO could be dem onstrated than in those in whom PPLO could not be demonstrated (Jensen 1954)

Other pathologic conditions of the male genitourinary tract from which PPLO have been isolated are fusospirochetal gangrene of the penis by Ruiter and Wentholt (1950) phagedenic ulcer on a young man with para

genicity of the strains of PPLO from man is fraught with difficulties It is the usual finding that PPLO exhibit pathogenicity by experimental inoculation into only the species of animal from which the organisms were isolated or at the most, there is a limited range of susceptible hosts Also many of the common laboratory animals are carriers of their own types of PPLO However there are a few instances in which certain strains of PPLO from man have shown some pathogenicity for experimental animals Two strains of PPLO were isolated by Ruiter and Wenthold in 1952 from cases of balanitis These differed from strains of PPLO isolated from cases of urethritis in colony morphology and cultural properties and were designated as G strains Inoculation of these organisms into the plantae of the hind legs of young white mice produced pathologic changes in the tibiotarsal joints from which the organisms could be cultivated In one case the knee joint and its surrounding musculature were involved Virulence of the organism was enhanced by serial passage through the mice No reaction could be elicited in the 2 patients when broth cultures of the 2 strains were injected intradermally Likewise no reaction occurred when a washed suspension of the organisms was introduced into the preputial pouch of 2 healthy men In a case of phagedenic ulcer associated with paraphimosis the intradermal injection of the necrotic material into the skin over the abdomen produced superficial ulcers From these fusiform bacilli and spirilla but no PPLO could be demonstrated Another G strain of PPLO was isolated from a case of fusospirillary vulvovaginitis and was similarly slightly pathogenic for young white mice Two strains of PPLO isolated from men with urethritis failed to show pathogenicity when injected subcutaneously into the pads of the hind feet of young white mice Additional evidence of the properties of communicability and pathogenicity of PPLO for man is the report by Kuzell and Mankle (1960) mentioned above and the infecting of human volunteers with *M pneumoniae* by Chanock Rifkind *et al* (1961)

Other Animals

Cattle The causative organism of bovine pleuropneumonia was cultivated by Nocard and co workers in 1898 and studied by Bordet and by Borrel *et al* in 1910 This organism is the type species and the name *Mycoplasma mycoides* var *mycoides* has been proposed In 1950 Edward isolated 2 groups of pleuropneumonialike organisms from the genital tract of cattle One group of strains provisionally designated P strains for which the name *Mycoplasma bovigenitalium* has been proposed was thought to be capable of causing an inflammation of the bovine genital tract which predisposes to infertility The strains were antigenically heterogeneous but they shared some antigens in common They were antigenically distinct from the S strains and the organism of pleuropneumonia Sera from infected animals did not show agglutinins for these strains The other group of strains isolated from the genital tract of cattle provisionally designated S strains resembled the saprophytic PPLO in not requiring serum for growth and in growing at room temperature They reacted serologically with the saprophytic strains from sewage were antigenically heterogeneous and were not related antigenically to the P strains or to the organism of pleuropneumonia It was thought that these strains were commensals or contaminants in the discharges PPLO and various species of bacteria have been isolated from the lungs of calves with the bronchopneumonia associated with the disease commonly referred to as shipping fever (Carter 1954) but the etiologic role of these organisms remains to be proved

Man may have more intimate contact with strains of PPLO from bovine sources than is generally realized as Alstrom (1955) found these organisms present in the milk from diseased and supposedly healthy cows in herds in which there was clinical mastitis Furthermore these organisms were reported to be resistant to pasteurization

Mice A communicable disease among white mice designated infectious catarrh was described by Nelson in 1937 as being caused by coccobacilliform bodies The similarity between the coccobacilliform bodies and PPLO is so close that in 1950 Nelson stated that there is little reason for separating them The organisms involved in the catarrhal condition tended to multiply throughout the respiratory tract particularly

ing beta hemolysis of guinea pig erythrocytes (Clyde 1963) in its nutritional requirements and antigenically (Taylor Robinson *et al* 1963) Like other respiratory pathogens the routes of transmission appear to be by aerosols and contact

PPLO have been cultivated from the lower end of the alimentary tract Harkness (1950) isolated PPLO 8 times from 45 individuals by means of anal swabs and swabs taken at sigmoidoscopy Morton *et al* (1952) isolated PPLO 3 times from feces from 27 individuals and Freundt (1953) isolated the organism from the rectal swabs of 7 of 25 women

No particular strain of PPLO from man has yet been demonstrated to have pyogenic properties as in the case of certain rat strains but PPLO have been associated with certain suppurative processes in man PPLO have been cultivated from abscessed Bartholin s glands in 6 cases a pelvic abscess associated with puerperal infection and a pelvic abscess associated with salpingitis (Dienes Ropes *et al*) a tubo ovarian abscess (Randall Stein and Ayres) and ovarian abscess in 2 patients and a periurethral abscess in a female patient (Melen 1952) in addition to 2 cases of brain abscess

Only 1 case of isolation of PPLO from an infection of the skin has been reported This infection a mixed infection with PPLO spirillae and fusiform bacilli was in and around the umbilicus of an obese woman in whom PPLO were also present in a leukorrheal exudate The skin and the genital strains of PPLO differed in several respects (Ruiter and Wentholt 1955)

Reports of involvement of the human central nervous system have not been numerous Mention has been made of the isolation of PPLO from brain abscesses in an individual injured with the stem of a smoking pipe and in a child PPLO were the only organisms cultured from spinal fluid from the case of meningitis that developed in an infant with a sacral meningocele which ruptured during birth (Davis and Arnstein 1953)

Cultivation of PPLO from the blood has been reported in a few cases and in these instances the bacteremia usually has been associated with a pathologic condition in some other part of the body Carlson *et al* (1951) isolated the organisms from the blood cultures of 3 children Two of the children had purpura a splinter in the finger of one child developed into an indolent ulcer the second child experienced a human bite and also had an intussusception of the ileum The third child had a brain abscess and meningitis Slingerland and Morgan (1952) isolated PPLO from the bloodstream and cervical secretions during a febrile illness which had its onset 24 hours after childbirth The appearance of the organisms in the bloodstream at the onset of the acute illness their persistence in the blood for over 24 hours and their disappearance with recovery of the patient suggest that the organisms were the cause of the acute illness Stokes (1955) also reported a similar case of puerperal sepsis

Cultures of fluids from inflamed joints of individuals with PPLO in other regions of the body have yielded PPLO only occasionally as compared with the findings in animals Dienes Ropes *et al* cultured PPLO from the synovial fluids of 2 patients with Reiter s syndrome and Brown *et al* (1951) cultured the organisms only once from synovial fluids Kuzell and Mankle (1950) reported isolating PPLO from the joint fluid in 5 cases of Reiter s syndrome The organisms have also been isolated from knee joint fluid from 2 cases of polyarthritis in women (Butas 1957)

It is only natural that interest should be aroused in the possible presence of PPLO in the conjunctivae of cases of Reiter s syndrome the triad of symptoms of urethritis arthritis and conjunctivitis Frequently PPLO are isolated from the urethral discharges of patients with Reiter s syndrome and successful isolations of PPLO from fluids of arthritic joints are becoming more frequent PPLO were isolated by Kuzell and Mankle (1950) from the conjunctivae of 5 cases of Reiter s syndrome Two of 3 patients with Reiter s syndrome gave significant agglutinin titers with an antigen prepared from one strain of PPLO (Wallerstein *et al* 1946) With the currently improved methods of cultivation and of performing serologic tests the etiologic role of PPLO in Reiter s syndrome may now be investigated with better chances of success

The experimental determination of patho

noticeable effect the etiologic role of these strains for disease in sheep remains to be demonstrated The situation may be similar to that in which Beveridge in 1941 isolated PPLO from cases of foot rot in sheep but showed that the organisms had no causal relationship with the disease (Greig 1955)

Goats There are at least 2 and possibly 3 diseases of goats which are thought to be caused by PPLO Contagious agalactia was discovered by Bridre and Donatien in 1925 as being caused by pleuropneumonia organisms for which the name *Mycoplasma agalactiae* has been proposed (Edward and Freundt 1956) Later it was discovered (Longley in 1951 Durusan *et al* 1952) that a contagious pleuropneumonia of goats was not caused by a filterable virus as originally thought but rather by PPLO for which the name *Mycoplasma mycoides* var *capri* has been suggested The pleuropneumonias of goats and cattle are similar in their pathology and the organisms of the 2 diseases have similar cultural properties By means of the complement fixation test Edward in 1953 was able to demonstrate that the two species of organisms are serologically distinct but that the two may share common antigens More recently a highly fatal disease characterized by septicemia and arthritis was dected for the first time among dairy goats on the North American continent Goats sheep and a pig were susceptible but mice guinea pigs and a calf were resistant to experimental inoculation However the organisms were highly virulent to chick embryos (Yamamoto *et al* 1955)

Swine PPLO have been isolated from the turbinates of normal swine and swine suffering from infectious atrophic rhinitis (Switzer 1955) These organisms when inoculated intranasally into baby pigs frequently become established in the nasal cavity but do not produce gross atrophy of the nasal turbinates Inoculated intraperitoneally into young pigs the organisms produce fibrinous peritonitis pleuritis pericarditis and arthritis Carter in 1954 pointed out that these lesions resemble those of Glasser s disease and he too was able to reproduce them by the intraperitoneal injection of cultures of PPLO Pigs over 6 weeks of age were observed by Switzer to show milder lesions From 5 to 20 per cent of the inoculated pigs developed arthritis The organisms can be grown in embryonated chicken eggs with about one half the embryos developing lesions or dying The swine PPLO were found to be avirulent for mice guinea pigs rabbits chickens turkeys a calf and a lamb The organisms do not produce acid from glucose maltose sucrose lactose or mannite they will withstand 56 C for 30 minutes but not for 1 hour Swine PPLO probably play a secondary role to a virus in producing pig pneumonia (Carter and Schroder 1956) The name *Mycoplasma hyorhinis* was proposed for these organisms and their natural habitat appears to be the nasal cavities of swine

Dogs Shoetensack in Japan in 1934 isolated from dogs with a respiratory type infection organisms which were similar to the organisms that cause pleuropneumonia in cattle The name *Asterococcus canis* was suggested for these newly isolated organisms In 1951 Edward and Fitzgerald in England isolated PPLO from the vaginal smears of 54 per cent of the dogs examined at a kennel and from the semen of a dog being investigated for sterility and epididymitis There was evidence that the organisms could be transmitted venereally by the dogs These authors also isolated PPLO from the throat specimens of 74 per cent of the dogs examined On the basis of colony morphology practically all of the canine strains of PPLO could be classified into 3 types designated α β and γ This classification into 3 types was verified by serologic studies The α strains never were detected in throat specimens from the dogs but the 3 types were isolated from the vagina The 3 types were isolated once from one vaginal specimen Pathogenicity studies were not made The PPLO isolated by Greig (1954) from dogs in Canada with kennel cough produced little if any effect in dogs on intranasal subcutaneous and intravenous inoculation The names *Mycoplasma spumans* *M* *canis* and *M* *maculosum* have been proposed for the α β and γ strains isolated from dogs An interesting observation is that of Dienes in 1939 in which he isolated a *Flavobacterium* and L type colonies from the dog bite wound of a patient How the organisms were introduced into the wound remains unknown

in the middle ears and were referred to as the catarrhal strain In the same year Nelson also described an outbreak of conjunctivitis unaccompanied by involvement of the respiratory tract in a colony of white mice The organisms were referred to as the conjunctival strain as they exhibited marked differences from the catarrhal strain and also differed from the organisms described by Sabin A high percentage of adult mice carried these organisms in their eyes but only a very small percentage of the carriers showed an inflammatory reaction Transmission to the young was thought to be by parental contact after the eyes became opened and continued after weaning by direct contact with cage mates The organisms were also isolated from the conjunctivae of 8 per cent of wild house mice Organisms of the catarrhal type selectively localized in the genital tract after intraperitoneal injection of female weanling mice but organisms of the conjunctival type failed to survive in the abdominal cavity and produced no reaction in the mice (Nelson 1954) The growth of the catarrhal and the conjunctival strains of PPLO was greatly enhanced in the brains of Swiss mice but not in Princeton mice by the simultaneous intracerebral injection of mouse hepatitis virus obtained from Balb C mice The growth and the pathogenicity of the catarrhal and the conjunctival strains of PPLO were greatly enhanced in the brains of Princeton mice but not in Swiss mice by the simultaneous intracerebral injection of mouse hepatitis virus obtained from Princeton mice (Nelson 1957)

During the brain to brain transfer of *Toxoplasma* in mice Sabin in 1938 in the United States encountered a new infectious agent with neurolytic properties The intracerebral injection of the agent into mice produced a characteristic rolling of the mice on the long axis of the body Later in the same year Sabin demonstrated the agent to be PPLO which elaborated an exotoxin From the brain of one of 10 normal stock mice a strain of PPLO was isolated During the following year this strain was found to produce arthritis in practically 100 per cent of the mice into which it was injected intravenously or intraperitoneally Also in 1938 Findlay *et al* in England isolated from the brains of mice during the intracerebral passage of the virus of lymphocytic choriomeningitis a strain of PPLO designated L_5 which produced the characteristic rolling symptoms observed years earlier This strain and that of Sabin were identical serologically but differed in pathogenicity (Sabin, 1941)

PPLO have been isolated from the brain the conjunctivae, the nasal mucosa and the lungs of normal mice It is evident that mice frequently are carriers of PPLO and the organisms comprise a variety of immunologic and biologic types This can introduce complications in experimental work with mice such as the finding by Edward in 1947 of PPLO in the seed culture of *Rickettsia orientalis* which had been obtained by the intranasal passage of material in mice

Rats PPLO have been isolated from the lungs of rats exhibiting chronic bronchopneumonia or bronchiectasis (the L_3 organisms described by Klieneberger and Steabben in 1937 and 1940) but the investigators were unable to produce acute pulmonary disease with the cultures by injection The organisms were isolated in 1939, from rats exhibiting polyarthritis by Findlay *et al* and in 1940 by Nelson from rats showing an infectious catarrhal condition The pyogenic virus of the rat which was described by Woglom and Warren was identified as the L_4 strain of PPLO in 1939 by Klieneberger and Woglom and Warren The L_1 variant of *S moniliformis* has been found in rats independently of *S moniliformis*

Sheep The disease known as contagious agalactia was demonstrated by Bridre and Donatien in 1925 to be caused by microorganisms resembling those of pleuropneumonia of cattle Although agalactia is a systemic disease the joints the eyes and in lactating sheep and goats the mammary glands are affected The name *Mycoplasma agalactiae* has been proposed for the causative organism Recently strains of PPLO which are different from *M agalactiae* have been isolated from the lungs of sheep in North America (Greig 1955 Adler *et al* 1956) These strains were infectious and lethal for chick embryos but were not infectious for mice guinea pigs and rabbits Since the parenteral inoculations of the strains into a limited number of sheep were without

isolated from the nasopharynx of healthy guinea pigs by Klieneberger in 1935 and from abscesses in guinea pigs (Klieneberger 1939 Findlay *et al* in 1940)

Horses Theiss (1947) isolated the organisms from an aborted fetus vaginal secretions and uterine discharges and Ito (1961) reported isolating PPLO from horses

Cats Recently PPLO have been isolated from the conjunctivae of domestic cats (Yerasimedes and Smith unpublished observations)

Hamsters and Monkeys There is one report (Ito 1960) that PPLO have been isolated from hamsters Taylor Robinson *et al* (1963) cite unpublished data that a strain of PPLO which cross reacted serologically with human type 1 strains had been isolated from the orpharynx of a *Cercopithicus* monkey

Saprophytic Strains

PPLO were isolated by Laidlaw and Elford (1936) from all samples of raw sewage from 4 districts of London during the summer but not from London tap water or from fecal material of man rabbit rat or pig Optimum growth took place at temperatures of about 30° C in media with a reaction of about pH 8 0 and although a very nutritious medium was required enrichment of the medium with a native body protein was not essential for growth Similar organisms were isolated from soil manure and compost by Seifert in 1937 in Germany Saprophytic strains have been isolated also from the bovine genital tract The name *Mycoplasma laidlawii* has been proposed for these strains

PATHOGENESIS

Little is known about the pathogenesis of infection with PPLO in man The organisms are communicable under natural and experimental conditions they may initiate or contribute to a pathologic process and specific antibodies may be demonstrated as a result of their activity They are capable of an intracellular existence so that one may expect a latent type of infection which could give rise to remission of symptoms or could be activated by trauma or stress

The L_1 variant of *S moniliformis* is only slightly if at all virulent for mice whereas the bacillary form of *S moniliformis* is virulent (Freundt 1956b) This may indicate that in general PPLO may be expected to be less virulent than bacteria However some of the human strains of PPLO have been demonstrated to be slightly virulent for mice With organisms of slight virulence a lessened resistance on the part of the host will play an important part in determining whether or not an infection is established

When the organisms are introduced into the bloodstream as a result of trauma such as that accompanying childbirth sepsis occasionally develops Since growth of the organisms is inhibited by their reaction with antibodies it can be expected that they will remain in the bloodstream only until specific antibodies appear Contrariwise when the organisms are introduced into the central nervous system where the immune reaction of the tissue is poor infection may result In general it is to be expected that the organisms will be able to establish themselves on surfaces of the body or in foci where the environment is suitable for their multiplication This is evident from the reports of their isolation from abscesses and inflamed mucous membranes

Some strains such as those from the mouse show a predilection for certain tissues e g the respiratory tract the conjunctiva and the female genital tract It is not known whether any of the human strains show a similar specific tissue tropism

Mixed infections play an important role in enabling some strains of PPLO to proliferate in certain animal species and similar circumstances may be operating in man Mention has been made of the enhancement of the growth and the pathogenicity of PPLO in mice infected with mouse hepatitis virus Rolling disease a new disease of mice was produced by Findlay *et al* in 1938 at first only with a specific strain of PPLO (L_5) together with some other nonspecific agent The nonspecific agent could be either the virus of lymphocytic choriomeningitis or of yellow fever (neurotropic strain) and climatic bubo or a suspension of serum agar culture medium Later by enhancing the virulence of the L strain by rapid brain to brain transfer in mice rolling disease could be produced directly by injection of the cul

Avian Species One of the areas of very active research in the relation of PPLO to infectious diseases is the general field of poultry science A limited investigation demonstrated a serologic relationship between a strain of PPLO isolated from chickens and a strain isolated from man (Smith *et al* 1957) which indicates the advisability of studying the ecolgy of PPLO Based on the study of only one species, the name *Mycoplasma gallinarum* has been proposed However the relationship of this strain to those isolated from avian species particularly chickens and turkeys in the United States and Canada remains to be studied

In extended studies of coryza in fowl started in the early 1930 s Nelson cultivated coccobacilliform bodies which were later considered to be PPLO The coryza of slow onset and long duration (coryza II) was shown to be caused by the coccobacilliform bodies whereas the coryza of rapid onset and short duration (coryza I) was caused by *H gallinarum* Coryza III had a rapid onset and a prolonged course and was found to be a mixed infection with *H gallinarum* and coccobacilliform bodies These interesting studies were confirmed by Adler and Yamamoto (1956b) Fahey and Crawley (1956) likewise presented evidence which they believed proved that infection of chicks with PPLO was mild in nature and was indistinguishable from coryza II The organisms of chronic respiratory disease of chickens and of infectious sinusitis of turkeys are pathogenic for the developing chick embryo (Markham and Wong 1952)

It has been demonstrated that infected hens transmit their PPLO to the egg and thus infect the embryos The PPLO and the host have established a state of reasonably peaceful coexistence so that only a small percentage of the embryos and young chicks are killed The carrier chicks infect their pen mates during the growing period by means of aerosols or contact The infection remains chronic in the adult birds and a small percentage of the adults pass the organisms through the egg to perpetuate the infection (Crawley and Fahey 1957)

If an infection with the virus of chronic respiratory disease or the virus of infectious bronchitis is superimposed on the mild infection with PPLO a much more serious infection results To bring under control the severe chronic respiratory disease of chickens it is necessary to break the cycle of transmission of PPLO from hen to egg This is done preferably by the parenteral administration of antibiotics (Adler *et al* 1956) Thus in order to control the severe chronic respiratory disease in chickens it is necessary to bring under control one of the organisms involved in the mixed infection and the control of PPLO affords the most practical solution

A hemagglutination inhibition (HI) test has been devised as a diagnostic test for infection with PPLO in chickens and in turkeys (Fahey 1954) The PPLO strains which are associated with chronic respiratory disease in chickens and infectious sinusitis in turkeys appear to be antigenically homogeneous with respect to this test and identical in morphology as shown by electron micrographs The continuous administration of either chlortetracycline or oxytetracycline in the feed does not free chickens from their PPLO and the antibiotics either retard or completely suppress the production of HI antibodies (Fahey and Crawley 1955)

Pigeons with a mild respiratory disease have yielded PPLO in cultures of the nasal discharges (Mathey *et al* 1956) The organisms did not ferment any of several carbohydrates which were tested and they differed in antigenicity and pathogenicity for chickens and turkeys from strains of PPLO which have been encountered in these 2 species

The isolation of PPLO from air sac exudate from a parakeet suffering from mild aerosacculitis introduces the possibility that isolating psittacosis virus from these birds may be more difficult (Adler 1957) The parakeet strain of PPLO was different antigenically from strains isolated from chickens and turkeys The organisms multiplied in the developing chick embryo but produced no mortality

PPLO and a virus were isolated by Fahey in Canada from ducks exhibiting a chronic respiratory disease The duck strain of PPLO fermented glucose and maltose weakly but did not ferment sucrose mannite and lactose The role of these organisms in producing disease in ducks remains to be elucidated

Guinea Pigs PPLO designated L_2 were

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ture PPLO have been shown also to proliferate more readily in mice infected with ectromelia virus (Schauwecker, 1947) An infection with the virus of either chronic respiratory disease or infectious bronchitis superimposed on a mild infection with PPLO produced a more serious disease in chickens

DIAGNOSIS

The presence of PPLO in the human body can be demonstrated only by cultivating the microorganisms in vitro as described in a previous section The characteristic appearances of the colonies on solid media and of the growth in liquid media are usually distinct enough to warrant the tentative placement of the microorganisms in the pleuropneumonia group In addition to their morphology colonies of suspected PPLO may be further verified by staining in situ in agar blocks by the method of Dienes or in petri dish cultures by means of diluted Dienes stain A technic whereby the colonies can be fixed to a microscopic slide and freed of the culture medium was described by Clark *et al* (1961) An agar block containing PPLO colonies is transferred to a microscope slide with the colonies in contact with the slide the slide is then placed in warm water and the temperature raised until the agar block melts or falls off the slide The slide with the colonies fixed to it is washed air dried and stained with Giemsa s or another appropriate stain Cultures stained by Giemsa s or Wayson s method may be examined to differentiate further the organisms from bacteria To study further the etiologic role of a strain of PPLO isolated from a patient, complement fixation tests may be performed with the patient s serum collected during the acute and the convalescent phases of the infection as was done by Stokes (1955)

Special serologic methods have been developed in studying avian strains A slide agglutination test employing a specially prepared antigen (Adler and Yamamoto 1956a) and a hemagglutination inhibition test have been employed for diagnostic work Human strains of PPLO do not agglutinate erythrocytes

TREATMENT

The sulfonamides penicillin erythromycin polymyxin and bacitracin do not inhibit growth in vitro and are ineffective as therapeutic agents against the PPLO from man (Leberman *et al* 1950 Rubin *et al* 1954) Streptomycin shows greater and more uniform inhibitory action in vitro than does dihydrostreptomycin (Leberman *et al*) That PPLO can develop drug resistance was demonstrated when a streptomycin sensitive strain became streptomycin resistant during therapy (Paine *et al*) when 2 strains developed resistance in vitro (Melen 1952) and when a streptomycin resistant strain was discovered to be the cause of an outbreak of infectious sinusitis in turkeys (Fahey 1957) Chlortetracycline and chloramphenicol inhibit the growth of some but by no means all strains in vitro Oxytetracycline and neomycin have an inhibitory action in vitro and oxytetracycline appears to be the drug of choice in the treatment of infections with PPLO (Leberman *et al* 1952 Robinson *et al* 1952) Oxytetracycline and tetracycline also appear to be the drugs of choice in the treatment of infections caused by the avian strains of PPLO (Yamamoto and Adler 1956)

Prior to the antibiotic era various gold compounds were found to be effective in preventing PPLO arthritis and encephalitis in mice and rats (Findlay *et al* 1940)

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could be transmitted to volunteers by means of filtered respiratory tract secretions

Studies in the last few years (Marmion and Goodburn 1961 Chanock *et al* 1961a b Clyde 1961 Clyde Denny and Dingle 1961 Rifkind *et al* 1962 Chanock Hayflick and Barile 1962) have demonstrated the etiologic relationship of *M pneumoniae* to cases of primary atypical pneumonia having cold hemagglutinins

CLINICAL PICTURE

The incubation period varies from 1 to 3 weeks with an average of 12 to 14 days An exact clinical characterization of the disease and its various manifestations is impossible because of diagnostic difficulties which will be mentioned later However a reasonably reliable concept can be obtained from cases observed during epidemics and cases resulting from the inoculation of volunteers (Reimann 1938 Dingle and Finland 1942 Curnen *et al* 1945 Commission on Acute Respiratory Diseases 1946 1947a b Jordan *et al* 1951 Doherty 1957 Chanock *et al* 1960 1961a b Mufson *et al* 1961 Kingston *et al* 1961 Miller *et al* 1963)

The onset is usually gradual and insidious The clinical features are those of a mild to moderately severe infection and the early complaints may be referable to either the upper or the lower respiratory passages or to both or may be principally constitutional in nature Respiratory symptoms include throat irritation and cough the latter being the most frequent and characteristic feature Constitutional symptoms include headache malaise feverishness chilliness fatigue and anorexia Headache is frequently an outstanding complaint and is particularly distressing when the patient coughs The cough is dry and frequently paroxysmal during the first 3 to 5 days of illness Ultimately it is productive of sputum which is either mucoid or mucopurulent Blood streaked sputum occurs in less than 10 per cent of cases A variable percentage of patients experience chest pain which appears to be directly related to the severity of the cough is substernal in location and is described as a burning sensation or aching discomfort In contrast with bacterial pneumonia most patients do not appear to be very ill early in the disease a shaking chill is rare the pulse is slow in relation to the fever respiration is normal or slightly increased grossly bloody sputum and pleural pain are uncommon herpes labialis is seldom present

Examination usually reveals few strikingly abnormal physical signs Early in the course the patient appears to be mildly or moderately ill with little more than slight inflammation of the throat Minimal dullness to percussion and diminution of breath sounds may be present but seldom reflect the extent of infiltration demonstrated by roentgenograms Early in the disease the same is true of rales Later the most characteristic physical finding is the presence of fine subcrepitant sticky rales in the absence of signs of true consolidation As the disease progresses rhonchi or coarse rales may be heard Myringitis has been described in volunteers (Rifkind *et al* 1962)

Roentgenographic examination often shows evidence of pneumonia before physical signs are apparent the disproportion between signs of infiltration and degree of pulmonary involvement demonstrated roentgenographically being an almost constant feature The consolidation usually appears to be most dense at the hilum and is progressively less dense near the periphery The borders of the pneumonic lesions are irregular and extension seems to occur in patchy fashion along the course of the bronchovascular trunks The shadows may appear diffuse mottled feathery or in rare instances dense The lower lobes are involved most frequently although any area in the lungs may be affected In approximately 50 per cent of patients pneumonia is present in only one lobe Spread of the infiltration with resolution of the early lesions as later ones appear is not uncommon Thus the changes are consistent with the concept that the infiltrative process is one of peribronchial peribronchiolar and interstitial inflammation associated with focal atelectasis and edema The roentgenographic signs may be transitory for a few days or may be progressive they generally undergo slow resolution over a period of 1 to 3 weeks Residual nodular densities or prominent bronchovascular markings may persist for several weeks

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36

Mycoplasma Pneumoniae Infections

INTRODUCTION

Primary atypical pneumonia is an acute usually self limited respiratory disease characterized by constitutional symptoms, cough and pulmonary infiltration most readily demonstrated by roentgenogram Obviously these characteristics are not specific, and similar clinical features may be manifested by several bacterial viral and rickettsial diseases of established etiology such as tularemia adenovirus infections psittacosis and Q fever One form of this syndrome featured by pneumonia and the development of cold hemagglutinins appeared to be an entity Such has proved to be the case and a pleuropneumonialike organism now termed *Mycoplasma pneumoniae* (Chanock *et al* 1963a) has been established as the causative agent However pneumonia illnesses occur in which this agent or other known agents cannot be found, and such cases are still referred to as primary atypical pneumonia

HISTORY

The differentiation of mycoplasmal pneumonia and other forms of primary atypical pneumonia from the bacterial pneumonias was accomplished in the late 1930 s and early 1940 s but it is probable that some of the similar cases reported previously represented the same disease Sections of lungs removed from soldiers during the Civil War showed features resembling those noted in fatal cases in more recent years Perhaps some of the influenzal pneumonias of World War I also were instances of the mycoplasmal variety of primary atypical pneumonia Between 1930 and 1937 Arrasmith Gallagher *Bowen Allen and Scadding reported cases* in adolescents and young adults of pneumonitis characterized by a benign course and a lack of correlation between roentgenographic changes and physical signs In 1938 and 1939 Reimann Smiley and others described similar cases and classified the disease as a new entity *Subsequently with* differentiation aided by the effectiveness of the sulfonamide drugs and penicillin in the bacterial pneumonias and the occasional epidemic occurrence of this nonbacterial disease the clinical and the epidemiologic features of primary atypical pneumonia were *detailed by Kneeland and Smetana Long*cope Dingle and Finland and many others During World War II the disease occurred commonly especially among military personnel and the Commission on Acute Respiratory Diseases provided evidence regarding its etiology by demonstrating that pneumonia



Fig 2 (A) Bronchiole containing polymorphonuclear leukocytes in its lumen The epithelium is increased in thickness but is still intact The wall of the bronchiole is infiltrated with mononuclear cells Phloxinemethylene blue stain (× 100) (B) Swollen alveolar walls and alveoli containing exudate of desquamated lining cells and mononuclear cells Giemsa's stain (× 100) (Parker F Jr Jolliffe L. S and Finland M 1947 Primary atypical pneumonia report of 8 cases with autopsies Arch Path *44* 581)

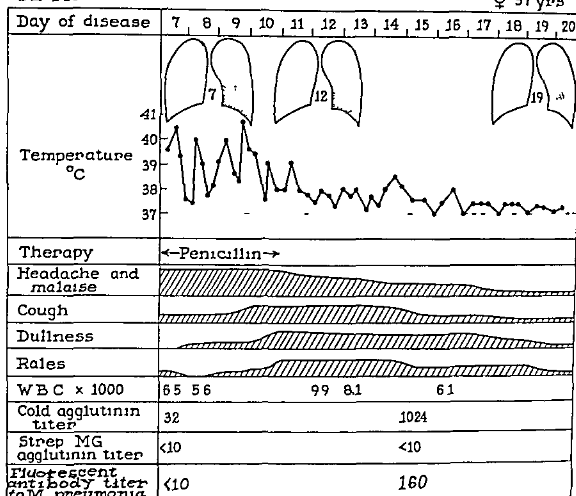


FIG 1 Chart of clinical findings in a case of mycoplasmal pneumonia

longer It is emphasized that the x ray picture is not distinctive or diagnostic and is simulated by other forms of pulmonary disease

Figure 1 illustrates the course of a case of average severity In this patient constitutional symptoms headache and cough were prominent and at the time of hospitalization on the 7th day there were no signs of consolidation and few rales despite demonstrable infiltration Eventually as happens in most instances this discrepancy tended to disappear and the extent of the physical signs became more comparable with the roentgenographic changes As in the case illustrated the majority of patients have normal total and differential leukocyte counts The count may become elevated as the illness progresses most often in association with spread of the infiltration in the absence of superimposed bacterial complications Erythrocyte counts and hemoglobin values are normal in uncomplicated cases The sedimentation rate is elevated The urine is normal Culture of the sputum shows bacterial species normally found in the respiratory tract *Mycoplasma pneumoniae* may be cultured from the throats and the sputa of these patients As noted subsequently a variety of immunologic reactions occur with sera of convalescent patients the most useful of which are the development of antibodies to *M pneumoniae* and of cold hemagglutinins Occasionally biologic false positive



FIG 2 (A) Bronchiole containing polymorphonuclear leukocytes in its lumen. The epithelium is increased in thickness but is still intact The wall of the bronchiole is infiltrated with mononuclear cells Phloxinemethylene blue stain (× 100) (B) Swollen alveolar walls and alveoli containing exudate of desquamated lining cells and mononuclear cells Giemsa's stain (× 100) (Parker F Jr Jolliffe L S and Finland M 1947 Primary atypical pneumonia report of 8 cases with autopsies *Arch Path* 44 581)

Wassermann or Kahn reactions may be obtained

The course of illness is extremely variable the duration of fever and the degree to which the temperature is elevated range widely *Fever may be present from 1 day to 6 weeks* it may be sustained but is most often remittent it usually terminates by lysis in from 7 to 14 days During the illness headache cough and sweating are commonly the most distressing symptoms With extensive pulmonary infiltration dyspnea cyanosis and abdominal distension occur In severely ill patients the infection may aggravate or precipitate cardiovascular dysfunction including auricular fibrillation decompensation or circulatory collapse However such an event is rare accordingly the case fatality rate is low (0 1 to 0 2 %) (Dingle *et al* 1944) Resolution begins with return of the temperature to normal and convalescence is usually uneventful except for asthenia

Complications are uncommon but involvement of nearly all organ systems has been reported myringitis sinusitis dermatitis pericarditis myocarditis encephalitis polyneuritis and thrombophlebitis Ulcerative tracheobronchitis may persist but a residual of true bronchiectasis is apparently rare although follow up studies have not been carried out extensively enough to permit a reliable appraisal of residual complications Acute hemolytic or embolic reactions may occur in certain patients with high titers of cold hemagglutinins an event usually induced by chilling Secondary bacterial infection is very uncommon

PATHOLOGIC PICTURE

No fatal cases have been reported since the nature of the causative organism was established The original Mac strain of *Mycoplasma pneumoniae* was isolated from the lung of a fatal case in California (Eaton *et al* 1944) One (*Sil*) *of several strains grown* in chick embryos in Boston in 1954 to 1955 (Liu 1957) and shown by immunofluorescence to be identical with the Mac strain was isolated *from the frozen lung specimen* of a patient who died in 1943 This patient was case 7 of a series of 8 fatal cases of cold hemagglutinin positive pneumonia with autopsies (Parker *et al* 1947) a fact that *provides presumptive evidence that the other* cases with similar pathologic pictures also succumbed to mycoplasmal pneumonia Because of the strong association of *M pneumoniae* infections with the development of cold hemagglutinins it is assumed that other fatal cases studied in the 1940s (Golden 1944) may be attributed to such infections Despite reservations regarding the lack of etiologic confirmation in these cases the changes described—in themselves not diagnostic—constitute the available information regarding the pathologic picture

Grossly the lungs are heavier than normal and areas of congestion and hemorrhage are apparent The pleural surfaces are usually normal but may show patches of fibrinous exudate Occasionally small amounts of straw colored pleural fluid are present The pneumonic areas may be extensive and widespread or discrete, circumscribed and multiple Nodular focal lesions resembling miliary granulomata may be present Various stages of consolidation as well as areas of atelectasis and emphysema are seen There is bronchitis and bronchiolitis, and the areas of infiltration are particularly prominent surrounding bronchi and bronchioles The lumina of these structures contain tenacious exudate The bronchial mucosa is inflamed and ulcerated

Microscopically, the picture is one of interstitial pneumonia and necrotizing bronchitis and bronchiolitis (Fig 2) The walls of the bronchi and the bronchioles show infiltration with mononuclear and polymorphonuclear cells which may *extend into the* peribronchiolar tissue and alveolar walls There is necrosis of epithelial cells with desquamation of the mucosa and ulceration The lumina contain *this debris mixed* with a purulent or mucoid exudate of mononuclear cells neutrophils and fibrin The alveoli show thickening of their walls dilation of the sep*tal capillaries edema in varying* degrees and infiltration of the septa by lymphocytes mononuclear cells and erythrocytes The alveolar spaces may be air-containing collapsed or partially filled with exudate and edema fluid Bacteria usually are seen in the pneumonic areas

Pathologic alterations in other organs have



FIG 3 A microcolony of *M pneumoniae* in rhesus monkey kidney cell culture The colony which measures 15 μ in diameter lies at the cytoplasmic junction of 3 cells whose deeply stained nuclei occupy the periphery of the figure The minute cocci composing the colony are just discernible at this magnification Intensified Giemsa × 1000 (From Dr W A Clyde Jr)

been observed particularly acute follicular splenitis mesenteric lymphadenitis focal hepatic necrosis acute myocarditis and hemorrhagic encephalitis

EXPERIMENTAL INFECTIONS HOST RANGE

The studies of the Commission on Acute Respiratory Diseases (1946) indicated that the disease could be transmitted and passed in volunteers under conditions suggesting a viral etiology When sera preserved from those experiments were studied using immunofluorescent technics (Clyde *et al* 1961) it was demonstrated that Eaton s agent now called *M pneumoniae* had been involved in these experiments After the agent had been grown in tissue culture but before it was known to be a PPLO infections were induced in volunteers with an inoculum grown in monkey kidney cells (Chanock *et al* 1961b) In both the earlier and the later groups of volunteers antibody responses were associated with cases of pneumonia and with a spectrum of respiratory disease ranging down to subclinical infection The more severe illnesses tended to occur in men without preinoculation antibody In both groups increases in titer of cold hemagglutinins and of streptococcus MG agglutinins developed in a significant percentage of volunteers mimicking serologic changes seen in natural infections Incubation periods of 9 to 12 days were consistent with those of the natural disease (Jordan 1949) Volunteers given the tissue culture propagated strain developed bullous myringitis (Rifkind *et al* 1962) a finding now described in naturally occurring infections Recently 2 strains of *M pneumoniae* grown on special PPLO medium were administered to 42 antibody free volunteers (Couch *et al* 1963) Detectable illness occurred in 23 febrile illness occurred in 8 none developed pneumonia or bullous myringitis The organism was recovered from 21 of 27 volunteers during 2 to 4 weeks after inoculation Thirty-eight of the 42 volunteers developed antibody to *M pneumoniae* as detected by immunofluorescence 23 developed complement fixing antibody Surprisingly in view of the mildness of the infections 31 developed a rise in cold hemagglutinins and 8 a rise in agglutinins for streptococcus MG It was suggested that propagation on a cell free agar medium resulted in a decrease in virulence a finding with implications for the development of attenuated strains to be used for immunization of man

M pneumoniae will replicate in a variety of tissue culture cells in chick embryos and in cotton rats guinea pigs and hamsters

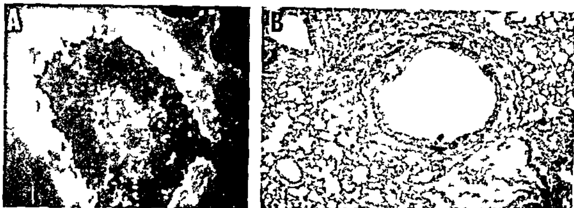


FIG 4 (A) Cross section of bronchus from a chick embryo infected with *M pneumoniae* The white area is a zone of intense fluorescence and represents the area to which antibody has been bound to antigen in the bronchial mucosa × 160 (Clyde W A Jr and Jordan W S Jr Diagnostic Procedures for Viral and Rickettsial Diseases ed 3 p 572 copy right by American Public Health Association New York 1964)

(B) Cross section of hamster bronchus 12 days following intranasal inoculation with broth culture of *M pneumoniae* Interstitial and peribronchial mononuclear cell infiltra tion extends through the muscularis of the bronchus × 35 (From Drs A S Dajani and W A Clyde Jr)

Growth without obvious cytopathology oc curs in monkey kidney cells (Chanock *et al* 1960b Clyde 1961) and chick entodermal cells (Gordon *et al* 1960) growth accom panied by cytopathic effects has been noted in continuous human amnion and lung cell lines (Eaton *et al* 1962) Extracellular mi crocolonies of minute cocci (Fig 3) are iden tifiable after infected tissue cells are stained (Clyde 1961, Eaton *et al* 1962) These clumps of minute granules react specifically with fluorescein labeled antibody Eaton (1962) has also noted intracytoplasmic mi crocolonies Coccobacilli line the bronchi of infected chick embryos in areas correspond ing to those which contain antigens localized by immunofluorescence (Fig 4 A) (Mar mion and Goodburn 1961) It was Liu s use of the immunofluorescent technic to detect such antigens which facilitated studies in chick embryos and cotton rats

Eaton *et al* (1942 1944) reported the production of pneumonic lesions in cotton rats and hamsters following primary intra nasal inoculation of sputa and lung suspen sions On serial passage the lesions either disappeared or apparently were replaced by lesions due to contamination or latent viruses The inoculation of chick embryos amniotic- ally with filtered sputum failed to produce any evidence of infection but lesions could be induced in cotton rats and hamsters inocu lated intranasally with 20 per cent suspen sions of these infected chick embryo tissues The agent (Mac strain) was maintained on repeated passage in chick embryos Liu (1957) demonstrated the transmission of in fection in the chick embryo using this strain and sputa and lung (Sil strain) from cases as indicated in two ways by the fluorescent antibody technic and by the induction of pulmonary consolidation in cotton rats fol lowing intranasal inoculation with suspen sions of chick embryo tissue Paradoxically, no lesion was recognizable at the site of the antigen in the chick embryo (Donald and Liu 1959) and no antigen could be detected at the site of the pneumonic lesion in the cotton rat Dajani and Clyde (1963) have undertaken to study the pathogenesis of in fection in hamsters inoculated with broth grown *M pneumoniae* using cultural meth ods now available to quantitate the growth of the organism in various tissues of the respiratory tract Interstitial and peribron chial polymorphonuclear and mononuclear cell infiltration with mononuclear elements predominating (Fig 4 B) similar to that seen in man has been induced and addi tional studies of antigen localization should

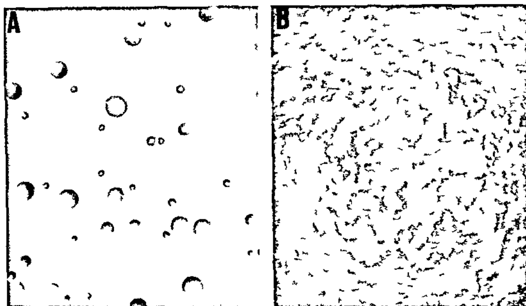


FIG 5 (A) *M pneumoniae* colonies after 1 week of growth on transparent agar The colonies appear as small spheres embedded in the agar matrix Colony diameter varies from 10 to 100μ Unstained $\times$ 400

(B) Week-old culture of *M pneumoniae* 24 hours after addition of sheep blood agar overlay Zones of beta hemolysis surround individual colonies and coalesce to form larger areas where colonies are closely spaced $\times$ 50 (From Clyde W A Jr and Denny F W Jr The Etiology and Therapy of Atypical Pneumonia Med Clin North Am *47* 1291)

clarify the nature of the experimental disease in this species

ETIOLOGY

In 1957 Eaton and Liu noted that the antibiotic spectrum of the atypical pneumonia agent corresponds most closely to some strains of pleuropneumonialike organisms or to rickettsiae and recalled that a search for PPLO had been made earlier by staining and culture methods in the regular passages of the agent Although the results were negative the suggestion that the agent might be a PPLO was revived when it was shown to be sensitive to gold salts and coccobacillary forms were seen in infected chick embryos (Marmion and Goodburn 1961) and in tissue cultures (Clyde 1961) That Eaton s agent is indeed a member of the *Mycoplasmataceae* was established when Chanock Hayflick and Barile (1962) successfully cultivated the organism in artificial media *Mycoplasma pneumoniae* has been proposed as a specific name for the organism (Chanock *et al* 1963a)

M pneumoniae shares the general properties of other mycoplasma as regards size shape lack of a rigid cell wall and ability to grow in a cell free environment Individual organisms are estimated by filtration to be 180 to 250 millimicrons in diameter (Eaton *et al* 1945a) Colony size is variable but seldom exceeds 0 5 mm Since the colonies grow into the agar they appear as spheres on the surface of a transparent agar medium (Fig 5 A) A surrounding halo formed by surface growth on the agar is usually not seen as opposed to the fried egg morphology of *M hominis* and *M salivarium*

Most mycoplasma have fastidious nutritional requirements cholesterol serum ascitic fluid or egg yolk being necessary for growth In addition *M pneumoniae* requires factors present in horse serum and fresh yeast

extract The organism ferments glucose, mal tose, dextrin, xylose, mannose and starch (Chanock *et al* 1963b) Glucose supple mentation enhances the growth of the organ ism in broth and the production of acid helps to distinguish it from other human my coplasmas with the exception of *M fer mentans* *M pneumoniae* grows more slowly on agar than other strains and colonies of naturally occurring strains may not be evi dent for 6 to 12 days Colonies develop more rapidly after 3 to 10 passages Growth in a broth medium also is slower than that of other human species maximum titers are reached by 9 to 16 days The organism will grow both aerobically and anaerobically A diphasic medium of equal parts of agar and broth incorporating a pH indicator, penicil lin and thallium acetate is commonly used for primary isolation attempts (Kenny *1963) Free floating colonies (10 to 100* microns) which produce a finely granular tur- bidity can be visualized by examining the tube at 50 to 100× magnification or by placing a drop of medium on a slide The broth is then subcultured on agar and the colonies tested for hemolytic capacity

The ability of *M pneumoniae* to lyse mam malian erythrocytes is a biologic property not shared by other human mycoplasma (Clyde 1963a Somerson *et al* 1963) Hu man horse guinea pig and sheep erythro cytes are lysed the cells of the last two species being the indicators of choice for preparation of an agar overlay to be poured onto culture plates bearing mature colonies The colonies produce β hemolysis after 24 to 48 hours (Fig 5 B) Each colony pro duces a hemolytic plague and absence of the organism from the peripheral area of hemol ysis suggests that the hemolysin is soluble No hemagglutinin has been detected Antigen may be adsorbed to tanned erythrocytes per- mitting use of an indirect hemagglutination test for measurement of specific antibody

Specific antisera and sera from patients convalescing from mycoplasmal pneumonia inhibit or neutralize the growth of *M pneu moniae* in cotton rats (Eaton *et al* 1945b) in tissue culture (Clyde 1963b) and in agar (Jensen *et al*, 1963) A complement fixing *antigen may* be prepared from broth cultures (Chanock *et al* 1962c) and the comple- ment fixation test has now replaced immu no fluorescent antibody staining for routine diagnostic purposes *M pneumoniae* does not share a group antigen with other human my coplasma Complement fixation and agar gel diffusion studies have shown minor anti genic relationship with other species (Taylor Robinson *et al*, *1963)*

The type of nucleic acid in the organism is not known with certainty Clusters of gran ules in tissue culture stain pink with May Grunwald Giemsa *but are resistant to de* oxyribonuclease and are Feulgen negative (Eaton *et al*, 1962) Coccobacillary bodies in the chick embryo give with acridine orange the green fluorescence characteristic of DNA (Marmion and Hers 1963)

Like all mycoplasma *M pneumoniae* is completely resistant to penicillin Before the organism was cultured successfully animal experiments indicated *that* carbomycin ery thromycin, tetracycline and chlortetracycline altered the course of infection Variable re sults were obtained with chloramphenicol and streptomycin (Eaton 1950, Eaton and Liu 1957) Clyde (1963b) has now pro vided quantitative sensitivity data from studies using infected tissue cultures At the range of antibiotic concentrations which are achieved in the sera of patients given recom mended doses the drugs which were effective against 10 000 organisms/ml were tetra cycline demethylchlortetracycline and olean domycin Streptomycin chloramphenicol chlortetracycline and oxytetracycline pro duced equivocal inhibition Kanamycin is effective against other mycoplasma (Pollock *et al* 1960) and presumably will be shown to inhibit *M pneumoniae* The organism is resistant to bacitracin and polymyxin B

DIAGNOSIS

Until *M pneumoniae* was established as the etiologic agent of an entity within the syndrome of primary atypical pneumonia as sociated with cold hemagglutinins the diag *nosis was based on a process of* exclusion Now confirmation is possible by means of practical and specific laboratory procedures (Clyde and Jordan 1964) The organism may be recovered from pharyngeal swabs or sputum using artificial media a specific sero

logic response may be demonstrated by the complement fixation test The agent may also be grown in cotton rats hamsters chick embryos and tissue culture cells and the immunofluorescent technic is still the most sensitive method of detecting antigen and measuring antibody For naturally occurring infections the complement fixation test has been estimated to be 80 per cent as sensitive as immunofluorescence with chick embryo lung sections (Chanock *et al* 1962c) In volunteers only 56 per cent of individuals with an antibody rise detectable by the fluorescent technic also developed a rise in complement fixing antibody (Couch *et al* 1963) However culture on agar or in broth and the complement fixation test are procedures most readily adaptable to the routine diagnostic laboratory Unfortunately growth of the mycoplasma and development of antibody require at least 1 and 3 weeks respectively *M pneumoniae* has been recovered from individuals with high levels of antibody when first seen suggesting that the agent may persist in the throat for a long time (Chanock *et al* 1962b) Hope for a rapid method of diagnosis rests with the demonstration by Hers *et al* (1963) that large mononuclear cells possibly representing desquamated alveolar or ciliated bronchial epithelium which may be stained by fluorescein labeled antibody to *M pneumoniae* are sometimes found in sputum

In addition to specific antibody response a variety of nonspecific immunologic reactions may be obtained with convalescent phase sera The most frequent and most useful are increasing titers of cold hemagglutinins and of streptococcus MG agglutinins Cold hemagglutinins for group O human erythrocytes appear in approximately 50 per cent of the patients with variations in different series of cases from about 30 per cent to almost 100 per cent Maximum titers are usually reached in the 3rd or the 4th week after onset following which a decline occurs during the succeeding 2 or 3 weeks In one group of patients with pneumonia cold hemagglutinins developed in 47 per cent of cases with antibody detectable by the fluorescent technic and in 7 per cent of cases without Agglutinins for streptococcus MG have been reported in some 20 to 75 per cent of cases

In the natural disease there is a positive correlation between the frequency with which both of these agglutinins develop and the severity of the disease the extent of pulmonary involvement and the duration of illness A similar correlation is found in the height of the titers reached Apart from mycoplasmal pneumonia cold hemagglutinins may be found in patients with trypanosomiasis and occasionally in patients with malaria blood dyscrasias or liver disease Agglutinins for streptococcus MG are rarely found in other patients Cold hemagglutinins and agglutinins for streptococcus MG appear to be different antibodies since each can be removed separately by adsorption of a serum containing both of them without appreciably affecting the titer of the other Both of them are distinct from the antibody for *M pneumoniae*

The differential diagnosis requires consideration of a number of infectious and noninfectious diseases that may present a similar or even an identical clinical picture Among the bacterial diseases tuberculosis tularemia and typhoid should be considered Of the fungal infections coccidioidomycosis and histoplasmosis are most likely to cause difficulty since these infections frequently occur without concomitant cutaneous involvement A history of residence in an endemic area is helpful in these cases Most of the rickettsial infections except Q fever can be recognized by the presence of an eschar or the development of a rash However Q fever may closely resemble mycoplasmal pneumonia although in general the onset is more sudden constitutional symptoms predominate throughout the illness respiratory symptoms are minimal and the roentgenographic evidence of infiltration is usually more focal in character and more likely to appear in the lung parenchyma without hilar involvement Although pulmonary infiltration may occur in such contagious diseases as measles and chickenpox the commonest viral diseases that should be considered in differential diagnosis are those due to the psittacosis group of viruses the influenza viruses the parainfluenza and respiratory syncytial viruses particularly in children and the adenoviruses A history of exposure to birds suggests the possibility of psittacosis Cases due to one of the influ

enza viruses or to adenoviruses are most likely to occur during epidemics of these diseases Rarely toxoplasmosis and ascariasis or other parasitic diseases must be considered in the differential diagnosis

In general, the final diagnosis of the above infections can be established by isolation of the infecting agent, by the demonstration of a rise in serum antibodies during convalescence or by both procedures Noninfectious processes resembling mycoplasmal pneumonia such as bronchiectasis eosinophilic pneumonia or Loeffler's syndrome sarcoidosis carcinoma, atelectasis and infarction usually can be recognized and differentiated by clinical and laboratory procedures and the course of illness

TREATMENT

A number of clinical trials had suggested that certain antibiotics particularly chlortetracycline, were effective in the treatment of patients with illnesses classified as primary atypical pneumonia but lack of specific diagnostic tests coupled with the natural variability of the infection made evaluation of these trials difficult (Clyde and Denny 1963) Measurement of antibody by immunofluorescence made diagnostic specificity possible and was the key to a field trial which resulted in findings compatible with the known inhibitory effects of tetracycline derivatives on mycoplasma (Kingston *et al* 1961) A double blind study compared the efficacy of demethylchlortetracycline and placebo therapy in 109 patients with pneumonia due to *M pneumoniae* and in 157 patients with pneumonia due to other causes It was found that 0 9 Gm of demethylchlortetracycline daily for 6 days significantly reduced the duration of fever (Fig 6), fatigue cough rales and pulmonary infiltration On the basis of sensitivity data (Clyde 1963b) tetracycline should be equally effective at the same concentration Although oleandomycin was even more potent in tissue culture assays potential toxicity precludes its use since other effective drugs are available

Knowing that tetracycline therapy is beneficial in patients with proved *M pneumoniae* infections has not made the physician's decision regarding institution of therapy any easier Since the available diagnostic technics take time, he must base his decision on the epidemiologic and the clinical features of a

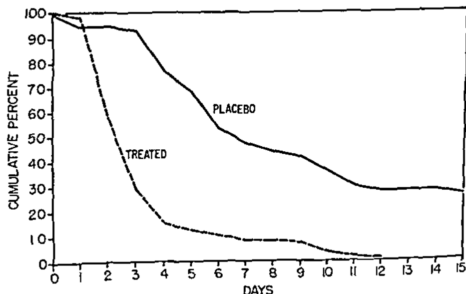


Fig 6 Duration of fever (99 F+) in Marine recruits with mycoplasmal pneumonia confirmed by fluorescent antibody response treated with demethylchlortetracycline or placebo (Adapted from Kingston *et al* 1961)

given case taking care to exclude bacterial and fungal diseases which might require different antibiotics Mild illnesses certain of which might be due to viruses should receive symptomatic and supportive treatment More severe cases should be given 0 5 Gm of tetracycline 4 times daily for 5 to 7 days Demethylchlortetracycline may be given according to the schedule used in the field trial (0 3 Gm 3 times daily) keeping in mind the photosensitizing effect of the drug Codeine or dihydrocodeinone will help to relieve headache and control cough Antipyretic drugs with a diaphoretic action such as acetylsalicylic acid should not be used if sweating is a prominent feature Sponge baths with cool water or alcohol should be avoided in severely ill patients because a hemolytic crisis may be induced if the titer of cold hemagglutinins in the patient s serum is high

EPIDEMIOLOGY

The disease occurs throughout the world usually in endemic form Its epidemic occurrence in both civilian groups (Gallagher 1934 Reimann 1938 Kneeland and Smetana 1940 Favour 1944 Jordan 1949 Johnson *et al* 1960) such as hospital personnel and families and in military populations (Campbell *et al* 1943 Dingle *et al* 1944 Kingston *et al* 1961 Miller *et al* 1963) has been described frequently and was one of the factors responsible for the recognition of this form of pneumonia Yet few epidemics have occurred in recent years and the cases observed since 1948 have been small in number and mild in nature Past behavior of the disease is crudely defined and the likelihood of future epidemics is totally unpredictable

The true incidence is not known Among armed forces personnel during World War II the attack rate averaged 10 per 1 000 per year about 10 times the incidence of bacterial pneumonia Use of specific diagnostic methods during 16 months of a smouldering epidemic at a U S Marine recruit depot demonstrated that *M pneumoniae* was associated with 56 per cent of atypical pneumonia and 28 per cent of febrile upper respiratory illness (Chanock *et al* 1961a) In contrast during an 18 month period at a military training center in the Netherlands 9 per cent of patients with acute respiratory disease not associated with influenza or adenovirus infection or 4 7 per cent of all cases were associated with *M pneumoniae* antibody increases In some epidemics attack rates as high as 15 to 20 per cent have been reported and in one study of multiple cases in families a rate of 35 per cent was noted (Jordan 1949) Case to-case spread has been apparent in such instances and the epidemiologic behavior has suggested a relationship between degree of contact and contagiousness The mechanism of transmission appears to be through direct contact with infected persons These inferences are supported by the results of studies in volunteers which indicated that the infection may be transmitted by respiratory secretions and that the portal of entry is the upper respiratory tract The duration of the period of communicability is unknown

An etiologic association between *M pneumoniae* and certain minor respiratory illnesses has been suggested by the sequence of occurrence of cases in epidemics and by transmission studies in man Such an association has been confirmed by specific serologic studies During the epidemic in Marine recruits referred to above 44 per cent of the men converted from a seronegative to a seropositive status during a 12 week training period there were 30 infections to every 1 clinical pneumonia (Chanock *et al* 1961a) Illustrative of variation with time and place is the observation that only 7 per cent of recruits in the Netherlands were infected with *M pneumoniae* during a 6 week period (van der Veen and van Nunen 1963)

The incidence of the disease usually rises during the winter months All age groups are attacked The degree and the duration of immunity following infection are not known but second attacks have been observed

CONTROL MEASURES

No specific prophylactic measure is available although the possibility of a specific vaccine is under study With the sporadic cases case to case spread is rarely apparent and isolation of the patients is seldom prac

ticed Isolation precautions may be helpful in the event of a spreading outbreak

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37

Medical Mycology

GENERAL

The *Actinomycetales* show a relationship not only to the single-celled bacteria but also to the higher filamentous fungi or molds They exhibit branching like the higher forms but their size is nearer that of the *Eubacteriales* They are all slender organisms their vegetative structures being 1 micron or less in diameter As found among the *Actinomycetaceae* they fragment readily into bacillary or coccoid elements or as found among the *Streptomycetaceae* they produce spores in the filaments as do the sporulating bacilli Also *Actinomyces* and *Nocardia* are sensitive to penicillin and sulfonamides respectively whereas the so called higher fungi are not affected by these agents Because of these characteristics the genera *Actinomyces Nocardia* and *Streptomyces* have been placed in the *Schizomycetes* in a position intermediate between the *Eubacteria* or true bacteria and the *Eumycetes* or true fungi

A true fungus on the other hand is characterized as having a simple plant body the thallus which lacks chlorophyll and is not differentiated into roots stems or leaves The thallus is composed of branching intertwined filaments or hyphae which form a dense mat of growth the mycelium Produced on or from the mycelium are various types of reproductive bodies or spores Spores differ greatly in their form and in the manner in which they are produced These two characteristics are used to classify the fungi

The relationship of the various groups of fungi pathogenic for man and animals is shown in Table 1

Organisms known to be pathogenic for man and animals are found in only a few of the groups shown in Table 1 In the *Schizomycetes* species of *Actinomyces* cause

TABLE 1

- Kingdom Plantae
  - Division Mycota
    - Subdivision Schizomycotina
      - Class Schizomycetes (Bacteria)
        - Order Actinomycetales
          - Family Actinomycetaceae
            - Genus Actinomyces
              - Species *A israelii*
              - *A bovis*
              - *A naeslundii*
            - Genus Nocardia
              - Species *N asteroides*
              - *N brasiliensis*
              - *N caviae*
          - Family Streptomycetaceae
            - Genus Streptomyces
              - Species *S madurae*
              - *S pelletierii*
              - *S paraguayensis*
              - *S somaliensis*
    - Subdivision Myxomycotina
      - Class Myxomycetes (Slime molds)
    - Subdivision Eumycotina (True fungi)
      - Class Phycomycetes (Alga fungi)
      - Class Ascomycetes (Sac fungi)
      - Class Basidiomycetes (Mushrooms)
      - Class Deuteromycetes (Imperfect fungi)

actinomycosis, *Nocardia asteroides* causes systemic nocardiosis and *N brasiliensis N caviae* and species of *Streptomyces* cause actinomycotic mycetoma Few fungi of the class *Phycomycetes* are pathogenic, species of *Absidia Rhizopus* and *Basidiobolus* cause phycomycosis, mucormycosis in man Among the *Ascomycetes* only *Allescheria boydii* and *Leptosphaeria senegalensis* cause maduromycosis while *Piedraia hortai* forms hard black nodules on the hair of the scalp No pathogens are found in the *Myxomycetes* or *Basidiomycetes* The remaining fungi or 76 per cent are found in the class *Deuteromycetes* the *Fungi Imperfecti*

IDENTIFICATION

With few exceptions (*Actinomyces Nocardia Cryptococcus Saccharomyces* and *Candida*) the fungi do not lend themselves to the use of bacteriologic technics for their identification They are all gram positive They do not ferment sugars do not reduce nitrates cannot be characterized by tests such as coagulation of serum or milk or the liquefaction of gelatin etc Therefore they are identified by their morphology macroscopically by the type of colony formation, and microscopically by the type of spores produced on or from the mycelium Macroscopically there are 3 colony types yeast yeast like and filamentous The yeast colony is soft and bacterial like and is composed of single celled budding forms (*Cryptococcus* and *Saccharomyces*) The yeastlike colony is also soft and bacterial like but is composed not only of single-celled budding forms on the surface of the medium but also of hyphae which penetrate the medium (*Candida* species) The filamentous colony presents the appearance of a typical mold and is composed of branching hyphae some of which penetrate the medium vegetative mycelium and some of which project from the surface aerial mycelium The aerial mycelium is referred to as a reproductive mycelium when spores are produced by the hyphae The type of spore produced and the method of its formation together with the type of colony provide the morphologic characteristics by which fungi are identified

The several types of spores produced by fungi may be divided into the two categories shown below

Spore Types

I Sexual Spores—produced as a result of nuclear fusion
  1 *Ascospores*—spores produced in a sac, the ascus found in the *Ascomycetes*
  2 *Basidiospores*—spores produced from a club shaped structure the basidium found in the *Basidiomycetes*
  3 *Zygospores*—spores produced by fusion of two identical cells found in the *Phycomycetes*
  4 *Oospores*—spores produced by fusion of two unlike cells found in the *Phycomycetes*

II Asexual Spores—produced in on or by the mycelium without nuclear fusion
  1 *Thallospores*—spores produced by changes in the mycelium or thallus
    A *Blastospores*—spores produced by budding, *Saccharomyces Candida* and *Cryptococcus*
    B *Chlamydospores*—spores produced by cells in the hyphae changing into thick walled resistant structures may be found in all fungi
    C *Arthrospores*—spores produced by fragmentation of hyphae *Geotrichum* and *Coccidioides*
  2 *True Conidia*—spores supported by a definite structure the conidiophore found among the *Imperfecti*
  3 *Sporangiospores*—spores produced inside a swollen structure *sporangium* on the end of a sporangiophore *Phycomycetes*

Examination of Fungi

Since the fungi can be identified only by the type and the arrangement of their spores preparations must be made that will allow examination of these structures The usual smear and staining methods used for the bacteria are not applicable to filamentous cultures as they break up the hyphae disperse the spores and prevent proper examination A preliminary examination of a tube culture is possible by placing the tube on the microscope stage and using the low

power objective on the top of the slant or along its edge

Slide cultures for continuous microscopic examination are made by various methods For example sterile slides and cover glasses under which inoculated warm agar is run support growth when placed in a damp chamber (sterile Petri dish with moist towel ing or filter paper) Development of the fungus from the edge of the agar may be followed microscopically In such preparations growth is undisturbed and the arrangement of the spores can be studied carefully

Routine examination of yeast or yeastlike cultures should be made by emulsifying some of the growth in a drop of water on a slide and covering the preparation with a cover glass Filamentous fungi are examined by carefully teasing with needles some of the mycelium in a drop of mounting medium i e lactophenol cotton blue or glycerine and eosin and covering the preparation with a cover glass

AEROBIC ACTINOMYCETES (NOCARDIA STREPTOMYCES)

The aerobic actinomycetes of the genera *Norcardia* and *Streptomyces* are free living in nature and several of the species may be introduced directly into wounds to cause localized actinomycotic mycetoma *Nocardia asteroides* may be inhaled to cause a primary pulmonary disease nocardiosis with eventual metastases to any area of the body particularly to the subcutaneous tissues and the central nervous system The species which cause such diseases are classified among the bacteria because of their size 1 micron or less in diameter their staining reactions gram positive and/or acid fast and the tendency for the mycelium of some of them to fragment into bacillary or coccoid elements Furthermore they are so closely similar morphologically that it is necessary to compare physiologic and biochemical reactions not only for separation into genera *Nocardia* and *Streptomyces* but also for separation of species within the genera (Gordon and Smith 1955 Mackinnon and Artagaveytia Allende 1956 Gordon and Mihm 1957 1959 1962a b Georg *et al* 1961 Mariat 1962)

History

The first pathogenic aerobic actinomycete was described from *farcin du boeuf* in cattle by Nocard in 1888 and later was named *Nocardia farcinica* by Trevisan in 1889 The first pathogenic aerobic actinomycete found in human infection pseudotuberculosis with brain abscesses and meningitis was described by Eppinger in 1880 as *Cladothrix asteroides* and later named *Nocardia asteroides* by Blanchard in 1896 Recently Gordon and Mihm (1962b) have reported that these two organisms *N farcinica* and *N asteroides* are probably identical and the human species *N asteroides* has been retained because of its importance in medicine

Vincent in 1894 described *Streptothrix madurae* as an etiologic agent of actinomycotic mycetoma (Madura foot) Since this early publication only a few species of *Nocardia* and *Streptomyces* have been shown to be the etiologic agents of approximately 480 cases of mycetoma reported from widely scattered areas of the world (Abbott 1956 Destombes *et al* 1958 Brounst *et al* 1962 Mariat 1963 Orio *et al* 1963)

Morphology and Variation

According to the classification of Waksman and Henrici (1943) *Nocardia* are aerobic partially acid fast actinomycetes whose vegetative mycelium readily fragments into bacillary and coccoid elements *Streptomyces* are those aerobic non acid fast actinomycetes whose vegetative mycelium does not fragment rather multiplication is accomplished by conidia in chains from aerial hyphae Therefore the aerobic actinomycetes with the distinctions mentioned above are gram positive partially acid fast or non acid fast branching filamentous organisms 1 micron or less in diameter However such organisms isolated from disease in man or animals were placed in the genus *Nocardia* until it became evident that gross and microscopic variation was so great among isolates that some of the pathogens should be placed in the genus *Streptomyces* Examination of several isolates by Gonzalez Ochoa and Sandoval (1955) showed conidial chain formation in *N madurae N somaliensis* and *N pelletierii* Furthermore Gordon and Smith (1955) have shown that physiologic

characteristics could also be used to separate isolations and place them into either the genus *Nocardia* or the genus *Streptomyces* On morphologic and physiologic criteria therefore the pathogenic aerobic actinomycetes now include *Nocardia asteroides*, *N brasiliensis* and *N caviae* as well as *Streptomyces somaliensis* *S pelletierii* *S madurae* and *S paraguayensis* The first named species *N asteroides* is the recognized etiologic agent of systemic nocardiosis while the remainder are recognized etiologic agents of localized actinomycotic mycetoma

In systemic nocardiosis, *N asteroides* appears in sputum empyemic fluid, spinal fluid and pus from subcutaneous abscesses as loose scattered, branching or bacillary gram positive and/or acid fast elements Not infrequently the acid fast bacillary elements may be mistaken for tubercle bacilli in stained sputum smears In localized actinomycotic mycetoma however the organisms appear in tissues and exudates from draining sinuses as yellowish white red or black granules with or without clubs at the periphery

Cultivation

Aerobic actinomycetes grow readily at 37° C or room temperature on a variety of simple media such as beef infusion glucose agar Sabouraud s glucose agar Czapek s agar etc However, colonies are slow growing and it is necessary to wait for 3 to 4 weeks before a typical appearance is obtained in the so-called giant cultures Such cultures show a marked variation in odor gross appearance pigment production and texture on different media and even on the same medium On solid media the colonies are usually glabrous wrinkled or granular and resemble those of some acid fast bacilli Occasional strains produce an aerial mycelium which gives the colony a chalky or powdery appearance, but on transfer this character may be lost Pigmentation is produced best and is more constant on Czapek s agar and varies depending on the species from cream to yellow, orange ochraceous and pink to coral or brick red The texture may be soft moist and mucilaginous or hard, dry and granular On liquid media the species develop wrinkled surface pellicles with the medium remaining clear (Figs 1 and 2)

In vitro studies of chemotherapeutic agents on species of *Nocardia* do not parallel in vivo studies so far as *N asteroides* is concerned Strauss *et al* (1951) found sodium sulfadiazine to be less effective than some of the antibiotics in vitro but to be more effective in vivo

Biochemical Reactions

A few of the biologic properties of the aerobic actinomycetes remain constant when several strains of the same species are tested Gordon and Smith (1955) found that the genera *Streptomyces* and *Nocardia* could be separated by the following reactions hydrolysis of casein decomposition of tyrosine and acid production from lactose maltose xylose and mannose These reactions were positive for *Streptomyces* but negative for *Nocardia* Pathogenic species within these genera can be separated by additional tests (Mackinnon and Artavageytia Allende 1956 Bojalil *et al* 1959 Mariat, 1962)

Pathogenicity

Nocardia asteroides is pathogenic for the rabbit the guinea pig and the mouse However, isolates of this species vary greatly in their ability to produce infection (Waksman and Henrici 1943, Georg *et al*, 1961) Rabbits injected intravenously with a sufficiently large inoculum develop a generalized infection with the production of miliary abscesses throughout the entire body Intramuscular and subcutaneous injections result in local abscesses only Guinea pigs injected intraperitoneally usually show a diffuse peritonitis with abscess formation on the peritoneal surface Death results from a toxic effect of the inoculated material rather than from extensive invasion by the fungus Mice injected intraperitoneally with *N asteroides* suspended in 5 per cent hog gastric mucin die with sufficient regularity to be used for in vivo testing of chemotherapeutic agents (Strauss *et al*, 1951) Intravenous inoculation of mice with small inocula followed by intraperitoneal injection with 0 5 ml of a 5 per cent suspension of gastric mucin is said to be a rapid method for testing the pathogenicity of isolates of *N asteroides* and *N brasiliensis* (Mohapatra and Pine 1963) Occasional isolates of *N brasiliensis* are also



Fig 1 (*Top left*) *N asteroides* Colony on Sabouraud s glucose agar 12 days old (*Top right*) *N asteroides* from Sabouraud s agar Gram stain × 970 (*Bottom left*) *N brasiliensis* Colony on Sabouraud s glucose agar 12 days old (*Bottom right*) *N brasiliensis* from Sabouraud s agar Gram stain × 970

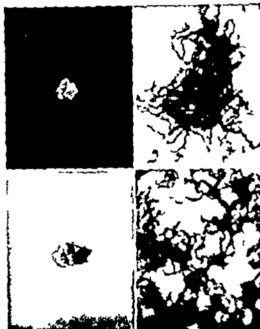


Fig 2 (*Top left*) *S madurae* Colony on Sabouraud s glucose agar 12 days old (*Top right*) *S madurae* from agar Gram stain × 970 (*Bottom left*) *S pelletieri* Colony on Sabouraud s glucose agar 12 days old (*Bottom right*) *S pelletieri* from Sabouraud s agar Gram stain × 970

pathogenic for the guinea pig (Ajello *et al* 1961) These organisms appear in gram stained smears of the pus from lesions in the infected animals as segregated loose mycelial fragments On occasion however it has been possible to produce granules in guinea pigs and mice with cultures of *N asteroides* and *N brasiliensis* when the inoculum was suspended in an adjuvant of paraffin oil or killed tubercle bacilli (Destombes *et al* 1961)

DISTRIBUTION

Nocardia asteroides is free living in nature has been described as an air borne laboratory contaminant (Henrici and Gardner 1921) and has been isolated repeatedly from soil (Gordon and Hagan 1936 Ajello 1956) Also *N brasiliensis* has recently been isolated from soil in Mexico (Gonzalez Ochoa and Sandoval 1960) Although other species of pathogenic aerobic actinomycetes have not been isolated from soil it is assumed that they exist as saprophytes in nature From this exogenous source the various species may be introduced into traumatized tissues to initiate localized chronic infections Therefore mycetoma is more prevalent in subtropical and tropical areas due to exposure of the body to the infectious organisms in the soil (Abbott 1956 Orio *et al* 1963 Mariat 1963) However systemic nocardiosis has a world wide distribution and is not a rare primary pulmonary infection due to inhalation of infectious *N asteroides* (McQuown 1955)

PATHOGENESIS

Systemic nocardiosis is caused by *N asteroides* and is chiefly pulmonary in origin (Murry *et al* 1961 Saltzman *et al* 1962 Freese *et al* 1963)

characteristics could also be used to separate isolations and place them into either the genus *Nocardia* or the genus *Streptomyces* On morphologic and physiologic criteria therefore the pathogenic aerobic actinomycetes now include *Nocardia asteroides*, *N brasiliensis* and *N caviae* as well as *Streptomyces somaliensis S pelletierii S madurae* and *S paraguayensis* The first named species *N asteroides* is the recognized etiologic agent of systemic nocardiosis while the remainder are recognized etiologic agents of localized actinomycotic mycetoma

In systemic nocardiosis *N asteroides* appears in sputum empyemic fluid spinal fluid and pus from subcutaneous abscesses as loose scattered, branching or bacillary gram-positive and/or acid fast elements Not infrequently the acid fast bacillary elements may be mistaken for tubercle bacilli in stained sputum smears In localized actinomycotic mycetoma, however the organisms appear in tissues and exudates from draining sinuses as yellowish white red or black granules *with or without clubs at the periphery*

Cultivation

Aerobic actinomycetes grow readily at 37° C or room temperature on a variety of simple media such as beef infusion glucose agar Sabouraud s glucose agar Czapek s agar, etc However colonies are slow growing and it is necessary to wait for 3 to 4 weeks before a typical appearance is obtained in the so-called giant cultures Such cultures show a marked variation in odor, gross appearance, pigment production and texture on different media and even on the same medium On solid media the colonies are usually glabrous wrinkled or granular and resemble *those of some acid fast bacilli* Occasional strains produce an aerial mycelium which gives the colony a chalky or powdery appearance but on transfer, this character may be lost Pigmentation is produced best and is more constant on Czapek s agar and varies depending on the species from cream to yellow orange ochraceous and pink to coral or brick red The texture may be soft moist and mucilaginous or hard dry and granular On liquid media the species develop wrinkled surface pellicles with the medium remaining clear (Figs 1 and 2)

In vitro studies of chemotherapeutic agents on species of *Nocardia* do not parallel in vivo studies so far as *N asteroides* is concerned Strauss *et al* (1951) found sodium sulfadiazine to be less effective than some of the antibiotics *in vitro* but to be more effective in vivo

Biochemical Reactions

A few of the biologic properties of the aerobic actinomycetes remain constant when several strains of the same species are tested Gordon and Smith (1955) found that the genera *Streptomyces* and *Nocardia* could be separated by the following reactions hydrolysis of casein decomposition of tyrosine and acid production from lactose maltose xylose and mannose These reactions were positive for *Streptomyces* but negative for *Nocardia* Pathogenic species within these genera can be separated by additional tests (Mackinnon and Artavageytia Allende 1956, Bojalil *et al* 1959, Mariat 1962)

Pathogenicity

Nocardia asteroides is pathogenic for the rabbit the guinea pig and the mouse However isolates of this species vary greatly in their ability to produce infection (Waksman and Henrici 1943 Georg *et al* 1961) Rabbits injected intravenously with a sufficiently large inoculum develop a generalized infection with the production of miliary abscesses throughout the entire body Intramuscular and subcutaneous injections result in local abscesses only Guinea pigs injected intraperitoneally usually show a diffuse peritonitis *with abscess formation on the peri*toneal surface Death results from a toxic effect of the inoculated material rather than *from extensive invasion* by the fungus Mice injected intraperitoneally with *N asteroides* suspended in 5 per cent hog gastric mucin die with sufficient regularity to be used for in vivo testing of chemotherapeutic agents (Strauss *et al* 1951) Intravenous inoculation of mice with small inocula followed by intraperitoneal injection with 0 5 ml of a 5 per cent suspension of gastric mucin is said to be a rapid method for testing the pathogenicity of isolates of *N asteroides* and *N brasiliensis* (Mohapatra and Pine 1963) Occasional isolates of *N brasiliensis* are also

laden macrophages enclosed by a fibrous capsule However diagnosis depends on the presence of granules surrounded by poly morphonuclear neutrophils centrally located in the abscesses (Fig 4)

Diagnosis

Sputum and pus from subcutaneous abscesses should be smeared and stained to demonstrate gram positive or acid fast branching hyphae Sputum without concen tration and pus may be inoculated on blood agar plates and Sabouraud s glucose agar slants for culturing at 37 C and room temperature respectively Spinal fluid should be centrifuged and the sediment stained for gram positive or acid fast hyphae and cul tured as for sputum and pus Antibiotics should not be added to the media since the aerobic actinomycetes are sensitive and may fail to grow

N asteroides obtained from clinical ma terials can be tested for pathogenicity by intraperitoneal injection of guinea pigs with saline suspensions of the fungus or by intra peritoneal injection of mice with 5 per cent hog gastric mucin.

Lesions of the subcutaneous tissues pro ducing the clinical picture of mycetoma should suggest infection by actinomycetes or infection by some of the higher fungi or molds (maduromycosis) Pus from the draining sinuses scrapings from the sinus walls and biopsy sections should be ex amined for actinomycotic granules Fresh preparations of the pus and scrapings should be prepared by placing a drop of the material on a slide and covering the preparation with a cover glass Microscopically the granules appear as amorphous lobulated masses sur rounded by pus cells The mass is composed of delicate (1 micron in diameter) tangled hyphae which may or may not be terminated by sheaths (clubs) at the periphery Such granules should be crushed and Gram stained When examined the smear contains short branching forms bacillary and coccoid elements which are gram positive Cultures are obtained by inoculating blood agar plates to be incubated at 37 C and Sabouraud s glucose agar slants to be incubated at room temperature with crushed granules

A purified polysaccharide obtained from *N brasiliensis* has been shown to be specific in an agar double-diffusion precipitin test with sera from patients infected with this organism No cross reactions occurred with sera from tuberculous patients from pa tients with mycetoma caused by *S madurae* or with sera from rabbits infected with *N asteroides* (Bojalil and Zamora 1963 Zamora *et al* 1963) Also purified proteins obtained from *N brasiliensis* elicited specific delayed skin tests in patients with mycetoma caused by this organism (Bojalil and Mag nusson 1963)

Treatment

Response to sulfonamide therapy is dra matic when systemic nocardiosis is diagnosed early High blood levels 15 to 20 mg per cent, should be maintained for 4 to 6 weeks during hospitalization of the patient lower blood levels should be maintained for at least 6 months after the patient s discharge Surgical drainage of cutaneous and other abscesses as well as empyemas may be necessary for effective drug therapy (Freese *et al* 1963)

The extent of infection in actinomycotic mycetoma will determine the effectiveness of therapy Surgical excision of the affected areas is necessary as an adjunct to chemo therapy The commonly used sulfonamides should be used for a period of months Also DDS and the prolonged action sulfonamides sulfamethoxypridazine and sulfadimethoxine have been used successfully (Mariat and Satre 1961)

Epidemiology

Pathogenic aerobic acid fast and non acid fast actinomycetes are free living in nature and cause disease by air borne con tamination or by introduction into tissues through trauma This view is substantiated by the study of systemic cases in which pul monary infection is the rule and of those cases demonstrating subcutaneous infection with no systemic reaction The possible ex tent of subclinical nocardiosis is not known However skin testing programs of large population groups with the antigens men tioned above may reveal the occurrence of widespread self limited disease

Many animals have spontaneous nocardi

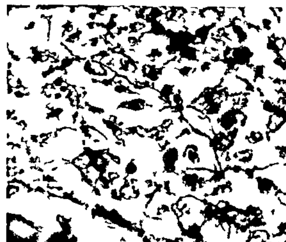


FIG 3 *Nocardia asteroides* Section of brain abscess Gram stain × 1 300 (Conant N F Smith D T, Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 41 Philadelphia Saunders)

Occasionally headache nausea and vomiting may suggest either brain tumor or brain abscess or the symptoms may be those of an infectious meningitis (tuberculous) with minimal or no findings in the lungs Symptoms referable to a pulmonary infection include general malaise fever productive cough with sputum night sweats anorexia and loss of weight Roentgenograms of the lungs usually show a progressive infiltrative process which may lead to multiple cavity formation Hematogenous spread results in metastatic lesions throughout the body Histologically, such lesions may be of a purulent nature, containing centers composed of polymorphonuclear neutrophils and a few mycelial fragments which can be demonstrated only when the sections are stained by Gram s method (Fig 3)

Actinomycotic mycetoma results in the clinical picture of Madura foot, although other subcutaneous tissues of the body also may become infected (Abbott 1956 Gonzalez Ochoa 1962 Orio *et al* 1963) The characteristic lesion with pain, swelling and sinus formation and eventual clubbing and marked deformity of the infected member is developed only after months or years Infection spreads by extension through adjacent tissues with bone destruction multiple abscesses which rupture and with no systemic reaction unless secondary bacterial invasion is established Histologically, sections of the sinus and abscess walls may show only a chronic inflammatory reaction Further development of the acute purulent abscess results in a surrounding layer of granulation tissue infiltrated with round cells and fat

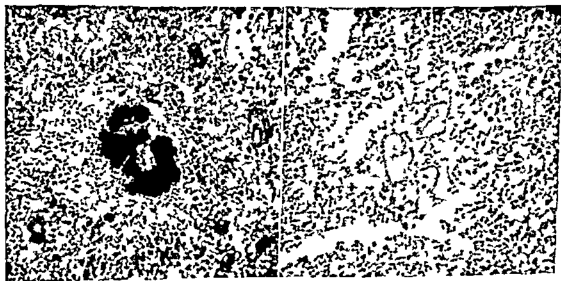


FIG 4 (*Left*) Granules of *S pelletieri* in subcutaneous tissue × 147 (*Right*) Granule of *N asteroides* in subcutaneous tissue × 147

glucose agar at room temperature the colony is glistening mucoid and tan to brown in color (Fig 5) Microscopically the cultures are best examined by emulsifying a portion of the growth in a drop of water or India ink under a cover glass Such preparations reveal thick walled ovoid to spherical budding cells 5 to 15 microns in diameter (Fig 6) surrounded by a wide gelatinous capsule no endospores are produced and no mycelium is developed

Cryptococci are nonfermenting yeasts which produce extracellular starch when grown on a defined medium containing dextrose and thiamine at a pH below 5 (Mager and Aschner 1946 Lodder and Kreger Van Rij 1952) Also all *Cryptococci* are urease positive (Seeliger 1956) Carbon and nitrogen assimilation tests are useful in distinguishing the single pathogenic species from other encapsulated closely related nonpathogenic species *C neoformans* does not assimilate nitrate (KNO_3) or lactose but does digest glucose maltose sucrose and galactose (Benham 1955) Its ability to grow at 37 C also distinguishes this species from nonpathogenic *Cryptococci*

The chemical nature of the capsular material of *C neoformans* has been studied by several investigators (Evans 1960) The capsular polysaccharide is composed of xylose mannose uronic acid and glucuronic acid Although Drouhet and Segretain (1949) reported a hyaluronic acid content based on destruction of the capsule by hyaluronidase this finding could not be confirmed (Foley and Uzman 1952 Littman and Zimmerman 1956)

C neoformans is pathogenic for the mouse when injected intraperitoneally or intracerebrally all other species of *Cryptococci* are nonpathogenic

In summary *C neoformans* can be identified by the following criteria (1) positive urease test (2) growth at 37 C (3) assimilation tests with KNO_3 lactose glucose maltose sucrose and galactose and (4) demonstrated pathogenicity for the mouse

In vitro studies have shown *C neoformans* to be resistant to most antibiotics and chemotherapeutic agents (Littman and Zimmerman 1956) However amphotericin B has been shown to be fungistatic in concentrations of 0 03 to 0 16 mg/ml (Louria *et al* 1957)

Distribution

Cryptococcosis was first called European blastomycosis but the world wide distribution of reported cases soon discredited ideas of geographic limitation *C neoformans* exists as a saprophyte in nature and produces disease in a variety of animals as well as man (Ajello 1958) The fungus has been isolated repeatedly from soil and is found in close association with pigeon nests (Emmons 1955 and 1960 Kao and Schwarz 1957 Yamamoto *et al* 1957 Littman and Schneierson 1959) There is no reported transmission from animal to man or man to man.

Pathogenesis

Cryptococcosis is a subacute or chronic infection caused by *C neoformans* which may affect the skin the lungs or other tissues of the body with almost invariable meningeal involvement terminally Clinically cutaneous and systemic types of infection have been described The cutaneous may be primary or may appear as a manifestation of an already established systemic infection Cutaneous lesions may appear as acneform pustules punched-out granulomatous ulcers subcutaneous tumors or deep-seated abscesses Many cutaneous cases progress to generalized infection with involvement of the lungs the visceral organs and the central nervous system Systemic cryptococcosis may involve the brain meninges lungs liver spleen pancreas thyroid and aorta In the majority of cases the central nervous system is the most frequently involved the lungs occasionally and other organs seldom Primary pulmonary infections resemble neoplasm or tuberculosis Brain infection may resemble an encephalitis acute or chronic meningitis of bacterial origin (especially tuberculous meningitis) brain tumor brain abscess central nervous system degeneration or central nervous system syphilis The spinal fluid pressure is increased, globulin and albumin increased cell count high chlorides and sugar content low

There is frequent association (10 to 30%) of cryptococcosis and the leukemia



Fig 5 *Cryptococcus neoformans* 12 days on Sabouraud s glucose agar at room temperature

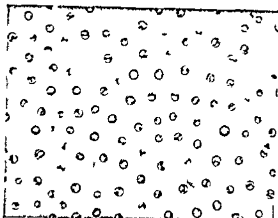


Fig 6 *Cryptococcus neoformans* round yeastlike cells with slight halo from Sabouraud s glucose agar at room temperature × 490

osis rabbit dog cat, pig goat, horse and cattle (Ajello *et al* 1961) However, there have been no reports of animal to man or man to man transmission of disease

CRYPTOCOCCUS NEOFORMANS

Cryptococcus neoformans is a yeastlike nonfermenting nonsporulating nonmycelial budding fungus characterized by the development of a wide capsule both in tissue and in culture It has a marked predilection for the central nervous system and produces a subacute or chronic infection of the meninges or lesions simulating brain tumor or brain abscess but may also involve the skin the lungs and other organs

History

There are many early reports concerned with budding yeastlike fungi which although given a variety of names and isolated from a variety of sources both human and animal are considered to be the same fungus *Cryptococcus neoformans*

A yeast *Saccharomyces* sp was described in Germany by Busse (1894 1896) and Buschke (1895) from a patient with localized subperiosteal infection of the tibia who later died with multiple lesions of the skin and viscera In Italy, Sanfelice (1894 1895a b) isolated a yeast from fermenting peach juice demonstrated its pathogenicity for laboratory animals and named the organism *Saccharomyces neoformans* because of its so-called tumor forming characteristics in experimental infections Also he reported (1895c) the isolation of a yeast *S lithogenes* from the lymph node of an ox with primary carcinoma of the liver Later (1898) he described *S granulomatogenes* isolated from the lung of swine Weis (1902) compared *S plimmeri* from cancer of the breast and *Torula sanfelice* from adenocarcinoma of a human ovary with *S neoformans* of Sanfelice and a yeast isolated from milk by Klein (1901) He placed these 4 organisms in the genus *Torula* Frothingham (1902) reported *Torula* sp from a tumor in the lung of a horse Later (1905) von Hansemann reported a yeast from the spinal fluid of a patient with suspected tuberculous meningitis and Versé (1914) reported leptomeningitis in a woman to be due to a yeastlike organism

In the United States Stoddard and Cutler (1916) reported 2 cases in man presenting signs of cerebral tumor which were caused by a budding fungus The fungus isolated from one of these cases was compared with that obtained by Frothingham in 1902 and was named *Torula histolytica* All these fungi are now considered to be identical and the yeast was named *Cryptococcus neoformans* after most of the above isolates were compared and reclassified (Benham 1935 Lodder and Kreger Van Rij 1952)

Cultivation

Cryptococcus neoformans may be cultured at room temperature or at 37 C on all common laboratory media On Sabouraud s



FIG 8 *Candida albicans* 20 days on Sabouraud's glucose agar at room temperature

of the material on a slide and gently pressing to a thin film under a cover glass Also these materials should be mixed with a small amount of India ink and examined under a cover glass before the preparation dries The fungus appears as a thick walled spherical budding yeastlike cell 5 to 15 microns in diameter surrounded by a wide capsule (Fig 7) Specimens should be cultured on Sabouraud's glucose agar at room temperature and blood agar at 37 C

A positive urease test immediately distinguishes *Cryptococcus* sp from *Candida* sp and true yeasts Ability to grow at 37 C and pathogenicity for the mouse distinguishes *C neoformans* from other similar but non pathogenic cryptococci

Treatment

A wide variety of drugs have been used to treat acute and chronic cryptococcosis (Littman and Zimmerman 1956) The chronicity of the disease with known remissions extending over a period of 12 or more years makes it difficult to evaluate any therapeutic regimen However successful treatment with amphotericin B has been well documented (Seabury and Dascomb 1958 Utz *et al* 1959 Newcomer *et al* 1960 Fitzpatrick and Poser 1960 Kress and Cantrell 1963 Barrash and Fort 1960 Smith *et al* 1960)

Epidemiology

Contaminated soil is the source of infection for both animals and man Rarely primary cutaneous infection can be established by direct inoculation of the skin The disease is established by inhalation of *C neoformans* with dust and dirt with metastasis throughout the body by lymphatic or hematogenous dissemination following a primary pulmonary disease The isolation of *C neoformans* from pigeon excreta in large cities would allow one to predict hundreds of subclinical infections within the population However the extent of infection has not been determined by skin testing surveys

CANDIDA ALBICANS

Candida albicans is an oval budding yeastlike fungus producing both blastospores and pseudomycelium in tissue and exudates and in culture at room temperature and at 37 C Its exact etiologic significance in any disease process is difficult to establish since it may frequently be found in the normal mouth and the intestinal tract or as a secondary contaminant in other recognized diseases

History

Langenbeck (1839) demonstrated in thrush the presence of a yeastlike budding fungus which Robin (1853) named *Oidium*



FIG 9 *Candida albicans* Pseudohyphae and clusters of blastospores on Sabouraud's glucose agar × 650 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 186 Philadelphia Saunders)

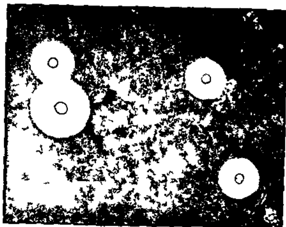


FIG 7 *Cryptococcus neoformans* India ink preparation of spinal fluid × 560

lymphoma group of diseases (Hutter and Collins 1962) Concurrent fungous infection may be due to the debilitated state of the patient who acquires exogenous infection or to activation of an existing latent infection The latter hypothesis is borne out by the incidental finding at autopsy of small subpleural nodules containing *C neoformans* in patients whose death was due to a variety of unrelated conditions (Haugen and Baker 1954) The use of steroid therapy urethane mustard gas and folic acid antagonists may activate such latent infections by *C neoformans* and a variety of other fungi (Zimmerman and Rappaport, 1954 Zimmerman 1955 Keye and Magee 1956 Baker 1962 Goldstein and Rambo 1962)

The histology of the lesions in the brain varies greatly some sections show only a minimal reaction with surprising lack of inflammatory cells other sections may show pseudotubercles formed of giant cells epithelioid cells and lymphocytes The centers of such lesions may be necrotic or hyalinized Lesions in other tissues particularly the skin are typical granulomata

Many early attempts to demonstrate antibody response by the patient to *C neoformans* have produced negative or conflicting results (Littman and Zimmerman 1956) However studies of antibody response in animals immunized with different strains have demonstrated capsular specificity of 3 serologic types designated A B and C (Evans 1949 Evans 1950, Evans and Kessel 1951) Also a capsular reaction has been reported with immune rabbit serum and *Cryptococcus* cells (Neill *et al* 1949) Such a reaction is not a quellung or swelling of the capsule since India ink preparations demonstrate the same size of capsule as does homologous serum (Evans *et al* 1956) Sera which are said to be useful for serologic identification of *C neoformans* have been obtained from rabbits (Tsuchiya *et al* 1963)

Infection in mice can be modified by immunization with *C neoformans* (Louria 1960 Louria *et al* 1963) Also treatment with immune rabbit serum gave longer survival time in experimentally infected mice (Gadebusch 1958)

Anticryptococcal sera have been shown to give serologic cross reactions with antigens from other fungi bacteria and gum tragacanth (Evans *et al*, 1953) The capsular material acidic polysaccharide apparently behaves as a polyvalent anion and as such reacts with a variety of cationic substances (Evans 1960 and 1962)

Soluble material in the spinal fluid the blood and the urine of a patient with cryptococcosis was found to precipitate in rabbit anticryptococcus serum (Neill *et al* 1951) Recently, antigen also has been detected in patients serum and spinal fluid by a slide agglutination test using antibody coated latex particles (Bloomfield *et al* 1963) Yeast cells and polysaccharide can be detected in tissues by the immunofluorescent staining technic (Eveland *et al* 1957) This technic also has been used to identify *C neoformans* in culture (Kase and Marshall 1960)

Various serologic technics have been used to detect antibody in the serum of patients with cryptococcosis—indirect fluorescent staining (Vogel *et al* 1961) hemagglutination test (Pollock and Ward 1962) and a complement fixation test (Bennett *et al* 1962)

A skin test antigen prepared from *C neoformans* has shown specificity in sensitized guinea pigs (Salvin and Smith 1961) Such an antigen would be useful for epidemiologic studies to determine the extent of subclinical infections

DIAGNOSIS

Sputum pus gelatinous exudates or sediment of centrifuged spinal fluid should be examined unstained by placing a small amount

TABLE 2 DIFFERENTIAL DIAGNOSIS OF SPECIES OF *Candida**

	PATHOGENIC	NONPATHOGENIC					
	C albicans	*C tropicalis*	*C pseudotropicalis*	*C krusei*	*C parapsilosis*	*C stellatoidea*	*C guilliermondii*
Sabouraud s agar	Creamy growth	Not characteristic	Not characteristic	Flat dry	Creamy	Creamy	Creamy growth
Sabouraud s broth	No surface growth	Narrow surface film with bubbles	No surface growth	Wide surface film	No surface growth	No surface growth	No surface growth
Blood agar	Medium sized dull gray colonies	Large gray colonies surrounded by mycelial frin e	Colonies small not characteristic	Colonies small irregularly shaped flat or heaped	Colonies small brilliant white	Colonies star shaped	Medium sized dull gray colonies
Corn meal agar	Branched treelike mycelium with chlamydospores	Mycelium well developed branched bearing numerous blastospores no chlamydo pores	Mycelium poorly developed no chlamydospores	Crossed sticks mycelium no chlamydospores	Mycelium well developed no chlamydospores	Mycelium with large ball like clusters of blastospores	Mycelium well developed no chlamydospores
Glucose	AG	AG	AG	AG	AG[1]	AG	[2]
Maltose	AG	AG				AG	
Sucrose	A	AG	AG				
Lactose			AG				

* Martin D S Jones C P Yao K F and Lee L E Jr 1937 A practical classification of the monilias J Bact *34* 99 1 9
[1] Occasionally acid only
[2] Langeron and Guerra report acid and gas produced in glucose and sucrose when cultured at 25 C and held 20 days

albicans The rudimentary morphology of the yeastlike fungi and the frequency with which they have been isolated from infected and contaminated materials has made classification and identification difficult However the application of physiologic biochemical and serologic technics to this group of organisms has allowed a classification to evolve that has proved to be useful Berkhout (1923) established the genus *Candida* and now the several species can be identified by standard laboratory methods

Candida albicans has been considered to be the only pathogenic member of the genus However other species are being isolated with increasing frequency from a variety of lesions in man

Cultivation

Candida albicans may be cultured on all common laboratory media both at room temperature and at 37° C On Sabouraud s glucose agar at room temperature the colonies are cream colored and soft and have a distinct yeastlike odor (Fig 8) The surface growth is composed of oval budding cells 2 5 × 4 to 6 microns while the submerged growth is composed of pseudomycelium This pseudomycelium in slide culture preparations is seen to consist of elongate, undetached cells with clusters of blastospores distributed at the points of constriction (Fig 9) On corn meal agar typical chlamydospores are produced (Fig 10) There is no surface growth on Sabouraud s glucose broth glucose and maltose are fermented with acid and gas, sucrose with acid only and lactose is not affected The common species of *Candida* can be differentiated by the technics of Martin *et al* (1937) Benham (1957) and Lodder and Kreger Van Rij (1952) The different species are presented in Table 2



Fig 10 *Candida albicans* Chlamydospores on corn meal agar × 750 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 186 Philadelphia Saunders)

Since *C albicans* is the species most frequently encountered in pathologic conditions and must be distinguished readily from saprophytic species found on or in the body several reliable quick methods for identification have been proposed It can be identified within 2 days by its colony morphology when grown on Levine eosin methylene blue agar under CO at 37° C (Weld 1953) Media to stimulate pigment production by incorporation of various compounds also has allowed rapid identification (MacLaren and Armen 1958) Other methods rely on a medium that stimulates chlamydospore production (Fig 10) which is the distinguishing morphologic characteristic of *C albicans* (Nickerson and Mankowski 1953 Taschdjian 1957 Taubert and Smith 1960 Gordon and Little 1963) Also rapid germ tube production from yeast cells suspended in serum distinguishes *C albicans* from other species (Taschdjian *et al* 1960)

Prolonged cultivation of *Candida* species in the presence of amphotericin B produces a slightly increased tolerance for the antibiotic (Littman *et al* 1958 Lones and Peacock 1959)

Distribution

Candida species are inhabitants of the normal mouth the intestinal tract and the vagina and may be cultured from these locations in 35 to 40 per cent of normal individuals Of this number about 15 to 20 per

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Sabouraud s broth	No surface growth	Narrow surface film with bubbles	No surface growth	Wide surface film	No surface growth	No surface growth	No surface growth
Blood agar	Medium sized dull gray colonies	Large gray colonies surrounded by mycelial fringe	Colonies small not characteristic	Colonies small irregularly shaped flat or heaped	Colonies small brilliant white	Colonies star shaped	Medium sized dull gray colonies
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Glucose	AG	AG	AG	AG	AG[1]	AG	
Maltose	AG	AG				AG	
Sucrose	A	AG	AG				
Lactose			AG				

* Martin D S Jones C P Yao K F and Lee L E Jr 1937 A practical classification of the monilias J Bact *34* 99 129

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Candida albicans has been considered to be the only pathogenic member of the genus However other species are being isolated with increasing frequency from a variety of lesions in man

Cultivation

Candida albicans may be cultured on all common laboratory media both at room temperature and at 37° C On Sabouraud s glucose agar at room temperature the colonies are cream colored and soft and have a distinct yeastlike odor (Fig 8) The surface growth is composed of oval budding cells 2 5 × 4 to 6 microns while the submerged growth is composed of pseudomycelium This pseudomycelium in slide culture preparations is seen to consist of elongate, undetached cells with clusters of blastospores distributed at the points of constriction (Fig 9) On corn meal agar typical chlamydospores are produced (Fig 10) There is no surface growth on Sabouraud s glucose broth glucose and maltose are fermented with acid and gas sucrose with acid only, and lactose is not affected The common species of *Candida* can be differentiated by the technics of Martin *et al* (1937) Benham (1957) and Lodder and Kreger Van Rij (1952) The different species are presented in Table 2



Fig 10 *Candida albicans* Chlamydospores on corn meal agar × 750 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 186 Philadelphia Saunders)

Since *C albicans* is the species most frequently encountered in pathologic conditions and must be distinguished readily from saprophytic species found on or in the body several reliable quick methods for identification have been proposed It can be identified within 2 days by its colony morphology when grown on Levine eosin methylene blue agar under CO at 37 C (Weld 1953) Media to stimulate pigment production by incorporation of various compounds also has allowed rapid identification (MacLaren and Armen 1958) Other methods rely on a medium that stimulates chlamydospore production (Fig 10) which is the distinguishing morphologic characteristic of *C albicans* (Nickerson and Mankowski 1953 Taschdjian 1957 Taubert and Smith 1960 Gordon and Little 1963) Also rapid germ tube production from yeast cells suspended in serum distinguishes *C albicans* from other species (Taschdjian *et al* 1960)

Prolonged cultivation of *Candida* species in the presence of amphotericin B produces a slightly increased tolerance for the antibiotic (Littman *et al* 1958 Lones and Peacock 1959)

Distribution

Candida species are inhabitants of the normal mouth the intestinal tract and the vagina and may be cultured from these locations in 35 to 40 per cent of normal individuals Of this number about 15 to 20 per

when stained by Gram s method such sections reveal the gram positive blastospores and the hyphal segments of *C albicans* in the centers of the tuberclelike structures and in areas of necrosis

Candidias occasionally accompany localized infections Such lesions are sterile and appear on the body as a result of sensitivity to the yeastlike fungi found in lesions elsewhere on the body

Although *C albicans* is considered to be the pathogenic member of the genus other species also have been isolated from disseminated infections Therefore definitive identification of a yeastlike fungus is necessary for a complete understanding of the pathogenesis of candidiasis



Fig 11 *Candida albicans* budding yeastlike cells in fresh preparation of sputum × 620

Immunity

The sera of patients with candidiasis will frequently show agglutination with saline suspensions of *C albicans* About 40 or 50 per cent of adults show a positive skin test to *C albicans* vaccine or oidiomycin (Lewis *et al* 1958) Both these tests have doubtful diagnostic significance The constant finding of *Candida* species on the skin in the mouth and in the intestinal tract of apparently normal individuals could account for both agglutinins and sensitivity to these fungi It has been shown (Drake 1945) that by a slide agglutination technic 45 per cent of normal human sera agglutinate *C albicans* These findings indicate that agglutination tests have little value However hypersensitivity should be considered and properly evaluated when treating a case of candidiasis

Serologic antigenic and infectivity studies in animals have proved to be useful for understanding not only the possible modes of reaction against infection by *C albicans* but also some of the pathogenic properties of this fungus Sera from immunized rabbits have been obtained for antigenic analysis of *Candida* species (Tsuchiya *et al* 1961) The single species *C albicans* has been shown to consist of 2 antigenic groups A and B (Hasenclever and Mitchell 1961) However several strains from each of these antigenic groups have the same degree of virulence for mice (Hasenclever and Mitchell 1961)

C albicans is pathogenic for mice on intraperitoneal injection when suspended in an adjuvant (Salvin *et al* 1952 Salvin 1952) Virulence is also enhanced in cortisone treated mice and in mice made Allaxon diabetic (Louria and Brown 1960 Andriole and Hasenclever 1962) However mice are uniformly susceptible to intravenous inoculation of *C albicans* but not other species when a suitable inoculum is used (Hasenclever 1959) Also soluble substances obtained from sonically oscillated cells have been shown to be lethal for mice (Mourad and Friedman 1961)

The pathogenicity of *C albicans* has been correlated with its ability to form mycelium quickly when inoculated into mice It is thought that the inability of polymorphonuclear cells to phagocytize the pseudomycelium results in dissemination of the fungus (Hill and Gebhardt 1956 Young 1958 Gresham and Whittle 1961 Louria and Brayton 1964)

Attempts have been made to use fluorescent antibody technics not only for identification and classification of yeastlike fungi but also as a tool for studying the pathogenesis of experimental infection in mice (Gordon 1962 Kemp and Solotorovsky 1962)

Diagnosis

Sputum and materials from lesions in the mouth and in the vagina should be examined as fresh cover glass preparations and as gram stained smears Skin or nail scrapings should be placed in a drop of 10 per cent KOH under a cover glass and the preparation

cent of the isolates have been identified as *C albicans* Thus, they are frequently isolated from pathologic materials with which they have no etiologic significance (MacKenzie 1961) With rare exceptions however *C albicans* has not been isolated from normal skin *C albicans* is found frequently in the sputum of patients with proved nonmycotic pulmonary disease (tuberculosis and carcinoma) It is also found in quantity in the stools of patients with diarrheal symptoms due to other causes (sprue and pernicious anemia) Many species of *Candida* including *C albicans* have been isolated from a variety of animals (van Uden and Carmo Sousa 1957) and from exogenous sources in nature (Ajello 1956)

Of the several yeastlike fungi of the genus *Candida* only *C albicans* is pathogenic for laboratory animals Rabbits injected intravenously with 1 ml of a 1 per cent saline suspension die in 4 to 5 days with typical abscesses in the kidneys Also mice are susceptible to an intravenous injection of 0 1 ml of a 1 per cent saline suspension

PATHOGENESIS

Candida albicans may cause infections of the mucous membranes of the mouth (thrush) and the vagina (vaginitis or vulvovaginitis) infections of the skin particularly of the intertriginous areas (axillae inframammary inguinal intergluteal interdigital webs of the hands and the feet) infections of the nails (onychia and paronychia) and systemic infections (bronchopulmonary or generalized infection of lungs lymph nodes liver spleen and meninges)

Infection of the mouth (thrush) is encountered more frequently in children than in adults In children it usually occurs following infection at birth from a mother with a vaginal infection (Taschdjian and Kozinn 1957 Kozinn *et al* 1960) In adults infection with *C albicans* usually follows a debilitating illness Such lesions appear as extensive or scattered whitish patches which contain the blastospores and the pseudomycelium of the fungus (Boggs *et al* 1961) Chronic oral lesions may last several years Occasionally the fungus spreads to the skin and the gastrointestinal tract to produce a generalized fatal candidiasis (Goldman and Schwarz 1962)

Vulvovaginitis, caused by infection of the vaginal mucosa and the vulva is a thrushlike infection characterized by irritation pruritus and a thin discharge

Infection of the skin usually occurs by autoinoculation of *C albicans* from the mouth or the intestinal tract Intertriginous lesions of the hand follow maceration of tissue by continued immersion in water Such lesions occur frequently in housewives waiters chefs bartenders fruit canners etc Intertrigo of the axillae and the intergluteal folds may become established because of obesity or diabetes Other intertriginous areas showing occasional infection are inframammary folds groin and interdigital webs of the toes Such lesions are characterized by erythematous exudative areas with well defined vesicopustular or papulosquamous borders

Infection of the nails (onychia and paronychia) is characterized by swelling at the nail bed which may be painful and resemble a pyogenic infection and by thickened transversely grooved nails

Infection of the eye (mycotic keratitis) follows trauma producing corneal ulceration which is difficult to manage (Manchester and Georg 1959)

Infections of the lungs may cause a mild bronchopulmonary candidiasis with persistent cough and with sputum containing cellular debris and yeastlike cells Roentgenograms reveal slight to moderate peribronchial thickening and scattered rales may be heard at the base of the lungs More extensive pulmonary candidiasis may resemble miliary tuberculosis with cough fever dyspnea chest pain hemoptysis and night sweats accompanied by signs of pleural thickening and consolidation

Systemic infection usually follows chronic debilitating disease prolonged use of wide spectrum antibiotics and steroidal compounds Frequently such infections may lead to involvement of many organs with terminal brain abscess meningitis and endocarditis (Louria *et al* 1962)

Histologically sections of skin may show abscess formation or a chronic inflammatory reaction with giant-cell formation Routine staining (H and E) may reveal tuberculoid granulomata with giant and epithelioid cells characteristic of tuberculosis However

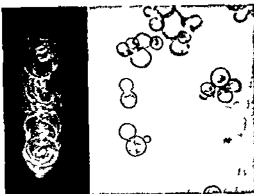


FIG 12 *Blastomyces dermatitidis* (*Left*) Yeastlike culture on blood agar 7 days at 37 C (*Right*) Yeastlike single budding cells from blood agar culture × 587

suppress or eliminate bacterial contamination (chloramphenicol 0 05 mg/ml and cycloheximide 0 5 mg/ml of medium) However the yeast phase growth of *B dermatitidis* may be suppressed in the presence of antibiotics when grown at 37 C (McDonough *et al* 1960) The duplicate culture at room temperature will overcome this difficulty and a positive culture will be obtained

Blastomyces dermatitidis may be grown at room temperature or at 37 C on all common laboratory media On blood agar or beef infusion glucose agar at 37 C the culture becomes wrinkled waxy and yeastlike in consistency (Fig 12) Microscopically it is composed of short broad 3 to 4-celled hyphal segments and budding yeastlike cells 8 to 20 microns in diameter similar to those seen in exudates or sections (Fig 12) On Sabouraud s glucose agar at room temperature the growth is at first smooth and yeastlike but quickly develops aerial projections and becomes prickly At this time a few budding cells may be found in such cultures but the majority of the growth has become filamentous A final overgrowth by white aerial mycelium which may turn brown with age establishes the completely filamentous stage of the fungus (Fig 13) Microscopically such a culture shows spherical to pyriform spores 5 to 8 microns in diameter attached directly to the hyphae or at the ends of short pedicles (Fig 14)

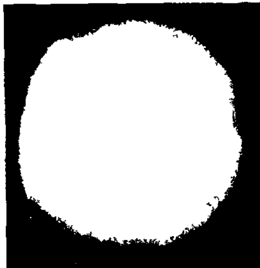


FIG 13 *Blastomyces dermatitidis* 21 days on Sabouraud s glucose agar at room temperature

This filamentous form can be converted to the yeastlike tissue phase by subculturing to blood agar and incubating at 37 C Temperature alone is responsible for the conversion of the mycelial to the yeast phase type of growth

ANTIGENIC STRUCTURE

There is one antigenic type of *B dermatitidis* as shown by the complement fixation



FIG 14 *Blastomyces dermatitidis* filamentous stage with conidia from Sabouraud s glucose agar culture at room temperature × 490

gently heated In fresh preparations *C albicans* appears as an oval budding yeastlike fungus 2 5 × 4 to 6 microns, with occasional hyphal fragments 2 5 × 6 to 12 microns (Fig 11) *C albicans* appears as gram positive oval budding, yeastlike cells and gram positive elongated hyphal cells

All materials should be cultured on Sabouraud s glucose agar at room temperature and at 37° C Clinical specimens should be streaked on Levine eosin methylene blue agar plates which are incubated at 37° C in a candle jar for rapid identification of *C albicans*

Antibiotics should be added to the media to prevent bacterial contamination (chloramphenicol 0 05 mg /ml)

Treatment

Oral and vaginal candidiasis may respond to alkaline mouth washes or alkaline douches, respectively In both cases a 1 per cent gentian violet (in 10 to 20% alcohol) used twice daily as a paint for 4 or 5 days has proved to be effective Sodium caprylate and the methyl and propyl esters of parahydroxybenzoic acid also have been recommended for oral and vaginal candidiasis A propionate vaginal jelly (propion gel) has been reported as giving excellent results in vulvovaginitis Carbowax sulfur ointment has been effective in the treatment of intertriginous areas vaginal candidiasis and paronychia However chronic oral or vaginal candidiasis may resist all therapy and eventually lead to dissemination The antibiotic nystatin has proved to be useful in the therapy of the conditions mentioned above

Also mysteclin a nystatin tetracycline compound has been used to avoid the effects of treatment with a wide spectrum antibiotic

Resistant and systemic candidiasis is treated best with amphotericin B (Louria 1958 Newcomer *et al* 1959 Louria and Dineen 1960 Kroetz *et al* 1962)

Epidemiology

Yeastlike fungi are found in the mouth in the intestinal tract in the vagina and on the skin of normal individuals The presence of a high percentage (40 to 50%) of positive skin reactions to oidiomycin or to an autogenous vaccine indicates that individuals may become hypersensitive to these organisms or their products Autoinoculation from any of the sites mentioned can cause disease especially during intensive antibiotic therapy

Control Measures

Patients with clinical candidiasis should be examined and treated for the presence of yeastlike fungi in the mouth or the intestinal tract to prevent autoinoculation

BLASTOMYCES DERMATITIDIS

Blastomyces dermatitidis is a spherical thick walled budding yeastlike fungus in tissue or exudates and in culture at 37° C In culture at room temperature it develops slowly as a typical moldlike filamentous fungus It produces a granulomatous infection of the skin and the internal organs very similar clinically and histologically to tuberculosis This disease is usually referred to as North American blastomycosis or Gilchrist s disease

History

Gilchrist (1896) first described blastomycotic dermatitis a disease resembling tuberculosis from biopsy specimens which showed refractive double-contoured budding cells in a section of skin A second case was reported by Gilchrist and Stokes (1896) from which they obtained a culture of the fungus which was named *Blastomyces dermatitidis* by Gilchrist and Stokes in 1898 Since these initial reports several fungi have been reported from North American blastomycosis namely *Glenospora gammeli Blastomycoides tulanensis Monosporium tulanense Endomyces capsulatus Endomyces dermatitidis* and *Glenospora brevis* A comparative study has shown them to be identical with Gilchrist s fungus *Blastomyces dermatitidis* (Conant 1939)

Cultivation

Clinical materials such as sputum pus from draining sinuses and biopsy specimens should be cultured on brain heart infusion glucose blood agar at 37° C and on Sabouraud s glucose agar at room temperature Antibiotics are used in the medium to

with coccidioidomycosis (Friedman and Conant 1953 Campbell and Brinkley 1953 Campbell 1960)

Diagnosis

Crusts from verrucous lesions should be placed in a drop of 10 per cent KOH under a covered glass and examined micro scopically after the preparation has been heated gently Sputum and pus from miliary abscesses at the border of cutaneous lesions or from subcutaneous lesions should be ex amined under a cover glass as untreated fresh preparations Spinal fluid and urine should be centrifuged and the sediment examined In all of these materials *B dermatitidis* ap pears as a thick walled single budding yeast like fungus 8 to 20 microns in diameter (Fig 15)

Culture materials on blood agar at 37 C and on Sabouraud s glucose agar at room temperature Heaped wrinkled isolated yeastlike colonies which appear on blood agar should be transferred to slants for fur ther development and identification

Treatment

Patients with systemic blastomycosis should receive supportive treatment a high calorie high vitamin diet with continued bed rest

The aromatic diamidines stilbamidine and dihydroxystilbamidine have been used successfully for the treatment of cutaneous and systemic blastomycosis (Curtis and Harrell 1952 Harrell *et al* 1955) Also amphotericin B has been used successfully in cases of blastomycosis which have failed to respond to the above drugs or have had periods of remission after adequate treat ment with them (Harrell and Curtis 1957 Newcomer *et al* 1959 Beard *et al* 1960)

Pulmonary resections and lobectomies have been performed with some success (Schwarz and Baum 1951 Buechner *et al* 1953 White and Owen 1954)

Epidemiology

Blastomycosis is a disease of the North American continent—the United States and Canada The etiologic agent *B dermatitidis* exists in nature but unlike *Histoplasma Coccidioides* etc it has been isolated from nature only once Essentially the dog is the only known animal to have spontaneous blastomycosis However there has been no report of animal to man transmission of the disease

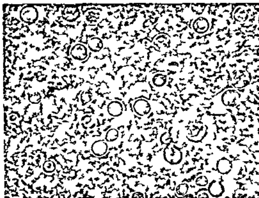


Fig 15 *Blastomyces dermatitidis* yeastlike single budding cells in pus × 475 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 54 Philadelphia Saunders)

Blastomycosis is a primary pulmonary in fection Few or many budding organisms can be demonstrated in the sputum and cultures are easily obtained However there have been no reports of man to man transmission of the disease

Until recently only sporadic cases of blas tomycosis have been reported In 1955 an epidemic of systemic blastomycosis involved 10 patients with the onset of the disease oc curring within a 5 month period (Smith *et al* 1955 Harris *et al* 1957)

BLASTOMYCES BRASILIENSIS

Blastomyces brasiliensis is a large thick walled single and multiple budding yeastlike fungus in exudates and tissues and in culture at 37 C In culture at room temperature the fungus develops slowly as a heaped cerebri form glabrous smooth colony or as a slow growing filamentous colony with a short white aerial mycelium It produces a granu lomatous infection of the mucous membranes of the mouth the lymph nodes and the in ternal organs The disease is known as South American blastomycosis paracoccidioidal

test (Martin, 1935) Soluble antigens from untreated and sonic treated yeast cells have been used in the complement fixation test and polysaccharides sensitize sheep cells to be used in the hemagglutination test (Martin 1953) Also Dyson and Evans (1954 1955), Knight and Marcus (1958) Knight *et al* (1959) and Marcus *et al* (1960) have reported antigens obtained by physical and chemical means from yeast cells to be active and specific when injected into the skin of humans and animals Gordon (1958) and Kaplan and Kaufman (1961) have used the fluorescent antibody technic to demonstrate specificity of *Blastomyces* antigens However they found that cross reactions occurred with *Candida albicans* and *Histoplasma capsulatum* Further investigations by Kaplan and Kaufman (1963) showed reaction of labeled globulin with the yeast phase but not with the mycelial phase of *B dermatitidis* A possible antigenic structure for the yeast cell antigens of *B dermatitidis* has been worked out by fluorescent antibody technics (Kaufman and Kaplan 1963) Ball *et al* (1960) used the agar gel diffusion precipitin test to demonstrate antibody in human serum

Distribution

So far as is known, *B dermatitidis* is confined to the United States and Canada A case reported from Europe in an American soldier could have been acquired before leaving this country (Brody 1947) The greatest incidence of blastomycosis in the United States is found in the eastern half of the country (Chick *et al* 1960) Many cases of spontaneous infection in dogs have been reported (Menges 1960) and also one in the horse (Benbrook *et al* 1948) and one in the sea lion (Weaver *et al* 1959) *B dermatitidis* has been isolated once from soil (Denton *et al* 1961)

Pathogenesis

Blastomycosis is a chronic granulomatous infection of the skin and the internal organs Primary cutaneous infection is rare and is characterized by an inflammation of the regional lymphatics which results in lymphadenopathy in association with the granulomatous or verrucous lesion (Schwarz and Baum 1951 Wilson *et al* 1955) Infection usually starts as a papulopustule which spreads peripherally showing a granulating base covered with a dirty pink exudate and a raised papilliform or verrucous border with miliary abscesses Spontaneous healing of the center produces scars of tissue paper thinness surrounded by the characteristic raised border (Abernathy 1959)

Primary pulmonary infection may be minimal or extensive and simulate tuberculosis or a malignancy With hematogenous dissemination the subcutaneous tissues skin and bones are most commonly affected Skin lesions appear anywhere on the body as multiple subcutaneous gummalike lesions which rupture spontaneously freeing bloody pus The vertebrae and the ribs are the bones most frequently involved

Lesions are also found in the central nervous system the liver the spleen and the kidneys where they are minimal and in the prostate The intestines are not affected (Kunkel *et al*, 1954 Weed, 1955, Cherniss and Waisbren 1956)

Two reports by Kunkel *et al* (1954) and Smith *et al* (1955) have shown that blastomycosis can occur as a primary pulmonary self limited disease similar to coccidioidomycosis histoplasmosis and tuberculosis

Histologically the lesions consist of numerous miliary abscesses containing polymorphonuclear cells cellular debris and giant cells Also tuberclelike lesions may be found which are indistinguishable from those seen in tuberculosis unless the fungus can be demonstrated

Immunity

Complement fixing antibodies can be demonstrated in the serum of patients with extensive or progressive infection These antibodies denote extent of infection and signify a poor prognosis They cannot be demonstrated in patients with localized cutaneous lesions (Smith 1949) Hypersensitivity to *Blastomyces* vaccine carbohydrate and protein fractions of the yeast phase and to blastomycin can be demonstrated in most cases a delayed tuberculinlike reaction becomes positive in 24 to 48 hours The antigens used also react in low titer with serum from patients with histoplasmosis South American blastomycosis and infrequently

many noncharacteristic hyphal swellings are seen in the mycelium On the aerial mycelium some strains develop round to pyriform sessile conidia 3 to 5 microns in diameter

Distribution

B brasiliensis is confined to the South American continent where hundreds of cases have been reported from Brazil and sporadic cases from other South American countries Three cases have been reported from Costa Rica in Central America (Trejos and Romero 1953) and one from Mexico (Gonzalez Ochoa and Esquivel 1950) One case has been described in a patient from the state of Oregon who had had previous residence in Venezuela (Perry *et al* 1954) There have been no reports of spontaneous infections in animals in the endemic areas nor has the fungus been isolated from soil

Pathogenesis

B brasiliensis causes a chronic granulomatous infection of the mucous membranes of the mouth and the adjacent skin of the face the lymph nodes and the viscera The mouth seems to be the portal of entry where ulcerating vegetative papillomatous lesions on the buccal mucosa spread to the adjacent skin of the lips and the nose These lesions resemble those of yaws or mucocutaneous leishmaniasis The infection may spread to the regional lymphatics and hence to the axillary inguinal and other nodes Occasionally direct infection of the lymph glands of the neck without demonstrable buccal lesions produces massive glandular enlargement similar to that seen in Hodgkin s disease A chronic type of cutaneous blastomycosis resulting in cheloid lesions has been described from the region of the Amazon This clinical variation is referred to as Lobo s disease In the visceral type of infection the intestines serve as the portal of entry Infection of the lymphoid tissue of the intestine with lymphatic drainage to the mesenteric nodes the spleen and the liver serves to establish massive visceral infection which simulates tuberculous peritonitis Hodgkin s disease or carcinoma of the abdominal cavity While the intestinal tract in South American blastomycosis shows gross infection it should be noted that the intestinal tract is never involved in North American blastomycosis

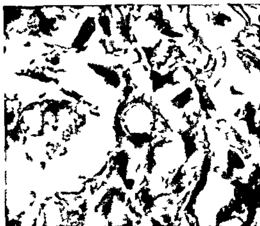


Fig 18 *Blastomyces brasiliensis* section of liver showing large thick walled cells with minute coccuslike buds × 1075

Pulmonary infection usually follows lymphatic or hematogenous dissemination of the fungus from lesions elsewhere in the body but may occur as a primary pulmonary infection caused by inhalation of infectious materials The lesions cannot be distinguished from tuberculosis Boeck s sarcoid carcinoma etc (Machado and Miranda 1960 de Paula 1962)

Histologically many areas show abscess formation with predominant polymorphonuclear infiltration other areas show focal lesions with necrotic and caseous centers surrounded by macrophages lymphocytes giant cells and fibroblasts The organisms appear in the tissue or giant cells as large round (10 to 60 microns in diameter) cells with small (1 to 5 microns) or large (10 to 30 microns) peripheral buds (Fig 18) This multiple budding is characteristic for *B brasiliensis* Frequent single budding forms 10 to 20 microns in diameter may be mistaken for *B dermatitidis*

Immunity

Positive complement fixation tests and positive intradermal skin tests have been reported in cases of South American blastomycosis As in North American blastomycosis a positive complement fixation test is

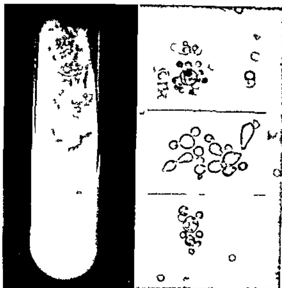


FIG 16 *Blastomyces brasiliensis* (*Left*) Yeastlike culture 12 days on beef infusion glucose agar at 37 C (*Right*) Multiple budding cells and moniliform cells from yeastlike culture at 37 C × 450



FIG 17 *Blastomyces brasiliensis* filamentous colony 28 days on Sabouraud s glucose agar at room temperature

granuloma or Lutz Splendore Almeida s disease

HISTORY

Lutz (1908) first reported pseudococcidioidal granuloma a localized disease of the mouth and regional lymphatics occurring in Brazil in which he described an organism thought to be closely related to *Coccidioides immitis* Carini (1908) reported a second case of localized buccal infection and called the disease blastomycosis The first generalized infection was described as blastomycosis by Splendore (1909) who later named the organism *Zymonema brasiliense* Haberfeld (1919) renamed this fungus *Zymonema histosporocellularis* while Arantes (1922) and Fonseca (1928 1929) described it as *Coccidioides immitis* However Almeida in 1930 compared cultures of *C immitis* with cultures from South American blastomycosis and found them to be different He named the South American fungus *Paracoccidioides brasiliensis* and Moore in 1935 1938 added 2 new species *P cerebriformis* and *P tenuis* Conant and Howell in 1942 reduced these species to synonymy with *P brasiliensis* and placed the South American fungus in the genus *Blastomyces* as *B brasiliensis* A cheloidal type of blastomycosis reported from the Amazon region by Lobo in 1931 and said by Fonseca and Leao in 1940 to be caused by *Glenosporella loboi* has been regarded as a different clinical entity occurring among natives in the Amazon but now known to occur in Central America

CULTIVATION

Clinical materials such as sputum pus biopsy specimens etc should be cultured on brain heart infusion blood glucose agar at 37 C and Sabouraud s glucose agar at room temperature Antibiotics may be added to these media to prevent overgrowth by contaminating bacteria (chloramphenicol 0 05 mg /ml and cycloheximide 0 5 mg /ml) On blood agar or beef infusion glucose agar at 37° C the culture becomes smooth waxy and yeastlike (Fig 16) It is composed of numerous round multiple budding yeastlike cells 6 to 30 microns in diameter with buds 1 5 to 5 microns in diameter scattered over the surface of the parent cell (Fig 16) Frequently single budding cells 8 to 14 microns in diameter are also seen These cells are identical with the yeastlike single budding forms seen in *B dermatitidis* There can also be seen short 2 to 4-celled moniliform chains from the cells of which multiple buds are produced (Fig 16) On Sabouraud s glucose agar at room temperature *B brasiliensis* grows slowly (1 5 to 2 cm in diameter in 3 weeks) forming a compact culture which may be smooth at first but later develops short aerial mycelium white to light brown in color (Fig 17) These cultures are composed of hyphae with short broad thick walled cells 2 to 3 × 4 microns in size which easily dissociate Numerous intercalary and terminal chlamydospores and

many noncharacteristic hyphal swellings are seen in the mycelium On the aerial mycelium some strains develop round to pyriform sessile conidia 3 to 5 microns in diameter

Distribution

B brasiliensis is confined to the South American continent where hundreds of cases have been reported from Brazil and sporadic cases from other South American countries Three cases have been reported from Costa Rica in Central America (Trejos and Romero 1953) and one from Mexico (Gonzalez Ochoa and Esquivel 1950) One case has been described in a patient from the state of Oregon who had had previous residence in Venezuela (Perry *et al* 1954) There have been no reports of spontaneous infections in animals in the endemic areas nor has the fungus been isolated from soil

Pathogenesis

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Immunity

Positive complement fixation tests and positive intradermal skin tests have been reported in cases of South American blastomycosis As in North American blastomycosis a positive complement fixation test is

indicative of a spreading infection It becomes negative after treatment with sulfonamides and is negative in minimal infections *Skin tests with the filtrate of* Sabouraud s broth in which several strains have been grown (paracoccidioidin) as well as with heat killed saline suspensions of the yeastlike phase of the fungus become positive in 24 to 48 hours Patients with South American blastomycosis frequently give a positive skin test and a positive serologic test with antigens from *B dermatitidis* (Lacaz 1956) Fava Netto (1955) extracted a polysaccharide from the yeast cells of *B brasiliensis* which gave a specific reaction in the complement fixation test Later, in 1961, he compared various antigens in the complement fixation and the precipitin tests In experimental infection in guinea pigs it was found that the delayed tuberculin type skin test became positive in 15 days while precipitin and complement fixation tests became positive in 15 to 30 days (Fava Netto *et al* 1961)

Diagnosis

Pus and scrapings from the buccal mucosa and the skin lesions pus from fluctuant nodes and smears of biopsied nodes should be examined as fresh preparations under a cover glass *Sputum in suspected pulmonary* infections should also be examined In such materials *B brasiliensis* appears as large (10 to 60 microns in diameter) round multiple budding cells The buds may be large and few in number from the surface of the parent cell and measure 10 to 30 microns in diameter or they may be small and numerous and measure 1 to 5 microns in diameter

Materials should be cultured on blood agar at 37° C and on Sabouraud s glucose agar at room temperature Guinea pigs may be inoculated intratesticularly with the infected materials and cultures obtained after the development of lesions

Treatment

South American blastomycosis responds dramatically to the sulfonamides but they must be given over long periods of time (Gonçalves 1962 Passos and Nahas 1959) Amphotericin B has been reported to be effective and can be used in those patients who show sensitivity to the sulfonamides (Lacaz and Sampaio 1958 Miranda and Machado 1959)

Epidemiology

South American blastomycosis is a disease of rural communities workers in close association with farming and soil showing a high incidence of infection Males are infected more frequently than females (10 to 1) except among the Japanese among whom the ratio is not so great *It is thought that* the Japanese women who work in the fields with the men have an equal opportunity for infection *Although it is felt that the fungus* must have a saprophytic existence in nature it has not been found in the soil or on natural substrata nor have infections of animals been reported

HISTOPLASMA CAPSULATUM

Histoplasma capsulatum is a small yeastlike oval intracellular fungus in tissues and is yeastlike on blood agar slants at 37° C In culture at room temperature it is a typical moldlike filamentous fungus *It is highly in*fectious it produces an acute benign self limited primary pulmonary infection and a chronic malignant progressive disseminated infection

History

Darling in 1906 first described an intracellular organism in the tissues from natives in the Canal Zone who had died of the disease similar to visceral leishmaniasis The organism was thought to be a protozoon closely related to *Leishmania donovani* and was named *Histoplasma capsulatum* Da Rocha Lima 1913 reported budding in these forms and called the organism *Cryptococcus* Hansmann and Schenken (1934) and De Monbreun (1934) were able to culture *H capsulatum* from their cases and proved it to be a filamentous fungus

Christie and Peterson (1945) and Palmer (1945) reported the existence of inapparent or subclinical infections based on skin sensitivity to *histoplasmin Until these reports* histoplasmosis was considered to be a highly fatal fungous infection

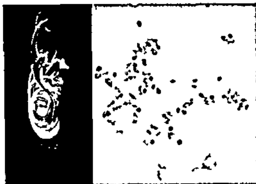


FIG 19 *Histoplasma capsulatum* (*Left*) Yeastlike growth on blood agar at 37 C for 5 days (*Right*) Small budding yeastlike cells from blood agar culture at 37 C × 520

CULTIVATION

Clinical materials such as sputum scrapings from lesions in the mouth and biopsy specimens should be cultured on brain heart infusion glucose blood agar at 37° C and on Sabouraud s glucose agar at room temperature Antibiotics are used in the medium to suppress bacterial contamination (chloramphenicol 0 05 mg /ml and cycloheximide 0 5 mg /ml)

Peripheral blood and bone marrow should be cultured as for bacteria in brain heart infusion glucose broth Slow growth is to be expected and these cultures should be held for 4 weeks before discarding

H capsulatum can be cultured on all common laboratory media On blood agar slants at 37 C the growth is yeastlike smooth white to cream colored and resembles *Staphylococcus albus* (Fig 19) It is composed of small (2 to 4 microns) oval single budding cells (Fig 19) On Sabouraud s glucose agar at room temperature it is cottony and white at first but becomes buff to brown with age (Fig 20) Young cultures show branching septate hyphae bearing small (2 5 to 5 microns) smooth round to pyriform spores on short pedicles Older cultures contain large (8 to 20 microns) round to pyriform, thick walled spores covered with finger like projections (Fig 21) These tuberculate spores are characteristic and diagnostic for *H capsulatum* The filamentous culture may

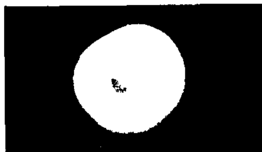


FIG 20 *Histoplasma capsulatum* 12 days on Sabouraud s glucose agar at room temperature

be converted to yeastlike tissue phase by subculturing to blood agar slants which are incubated at 37 C Such yeastlike cultures may be maintained by subculturing to fresh blood agar slants every 4 to 5 days

ANTIGENIC STRUCTURE

There is one antigenic type of *H capsulatum* Histoplasmin is a filtrate of a liquid culture medium in which the filamentous phase has been grown for varying periods of time It is probably carbohydrate in nature and elicits a delayed reaction in previously



FIG 21 *Histoplasma capsulatum* typical tuberculate chlamydospores developed in the Sabouraud s glucose agar culture at room temperature × 556 (Smith D T Am J Med 2 599)

infected animals or humans in intradermal injection This material also acts as a precipitating antigen in agar gel diffusion tests Heiner (1958) reported 6 bands representing 6 different antigens and their corresponding antibodies on testing various sera These bands were found to be nonspecific n specific h and cross reactive c This last band represents a product of *B dermatitidis* and *C immitis* as well as *H capsulatum* An m band represented a reaction that could be elicited by histoplasmin skin tests in normal sensitized adults The specific h band was isolated from histoplasmin by continuous flow electrophoresis Greene *et al* (1960) purified histoplasmin giving h and m bands by means of DEAE chromatography and were able to separate these 2 bands Schubert *et al* (1961) also reported only 2 bands h and m elicited by their histoplasmin Used in tests with 5 211 specimens of sera they reported the test to be presumptive of infection by *H capsulatum* A polysaccharide extracted from yeast cells of *H capsulatum* gave skin test reactions comparable to histoplasmin when tested in 3 808 individuals (Edwards *et al* 1961 Knight and Marcus 1958 Knight *et al* 1959)

Salvin and Ribi (1955) reported the yeast cell wall antigen of *H capsulatum* to be associated with serologic tests protection and toxicity It could also be used as an antigen for intradermal tests Cozad and Larsh (1960) used stained broken yeast cells in a capillary tube agglutination test while previously Cozad (1958) had used the whole yeast cell as an antigen in the agglutination test Collodion and latex particles sensitized with histoplasmin also have been used in an agglutination test (Saslaw and Campbell 1949 Saslaw and Carlisle 1958) Fluorescent antibody technics have been used for the detection of the yeast and mycelial phases of *H capsulatum* (Gordon 1959 Kaufman and Kaplan 1961 Kaufman and Brandt 1964)

Distribution

Histoplasmosis occurs throughout the world with areas of high endemicity as determined by histoplasmin skin testing programs (Edwards and Klaer 1956 Manos *et al*, 1956) Areas of endemicity in the United States include the northeast the central and the south central states (Edwards and Palmer 1963) It is estimated that from 25 to 30 million people in the United States have had some form of the disease (Loosli 1957) *H capsulatum* has been isolated from the soil and spontaneous infection in a variety of animals has been reported (Emmons 1949 Emmons *et al* 1955)

Pathogenesis

Histoplasmosis is a primary pulmonary infection caused by inhalation of the highly infectious *H capsulatum* The respiratory infection may be subclinical mild or severe and simulate infection caused by any bacterial or viral agent In the vast majority of cases the infection is benign self limited and inapparent and revealed only by a positive skin test to histoplasmin

Acute pulmonary histoplasmosis is accompanied by fever cough malaise sweating and loss of weight In the lungs scattered miliary multiple nodular or parenchymal lesions may simulate tuberculosis Such lesions may undergo complete resolution but characteristically heal by calcification in 4 to 5 years (Bronson and Schwarz 1957) A few of the pulmonary infections become chronic and progressive resulting in fibrocaseous lesions with cavity formation in the upper lobes (Furcolow and Brasher 1956 Loewen *et al* 1960) The residual lesion remaining after infection of the lungs coin shaped histoplasmoma is probably the most frequent of such lesions and must be considered in a differential diagnosis of carcinoma tuberculoma etc (Editorial 1960)

During the primary pulmonary infection the fungus may disseminate throughout the body by lymphatic or hematogenous spread to parasitize the reticuloendothelial system Symptomatic progressive disease apparently is dependent on the resistance of the infected individual Dissemination in the resistant individual has been demonstrated by a high rate of splenic calcification in known endemic areas of histoplasmosis (Schwarz *et al* 1955 Serviansky and Schwarz 1956) The nonresistant individual shows the typical symptoms of acute progressive histoplasmosis fever malaise sweats splenomegaly hepato

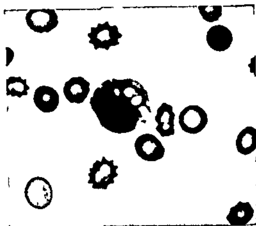


FIG 22 *Histoplasma capsulatum* peripheral blood smear showing *Histoplasma* in mononuclear cell × 1 155

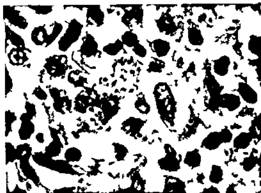


FIG 23 *Histoplasma capsulatum* section of gum showing intracellular bodies × 1 012

megaly leukopenia secondary anemia and emaciation Mucocutaneous lesions (skin tongue pharynx larynx) are manifestations of systemic infection with metastasis to the involved areas and occur frequently in chronic progressive infections (Baum *et al* 1957)

Histologically the lesions show a central necrosis with loss of tissue and cellular structures surrounded by granulomatous tissue The small 2 to 5 microns oval cells of the fungus appear in various phagocytic cells large mononuclear or polymorphonuclear cells of the blood and bone marrow endothelial wandering cells of tissues and fixed reticuloendothelial cells of the liver and the spleen

IMMUNITY

Van Pernis Benson and Holinger (1941) were the first to show that a patient and experimentally infected mice gave a positive skin test to filtrates of a broth in which *H capsulatum* had been grown A delayed tuberculinlike reaction became positive in 24 to 48 hours Thousands of skin tests have been done with histoplasmin on people living in areas where there is known to be a high rate of nontuberculous pulmonary calcification as determined by negative tuberculin tests indicating a high resistance to the fungus

Serologic response to infection appears during the 3rd or the 4th week Precipitins and complement fixing antibodies have been demonstrated (Salvin and Furcolow 1954 Loosli 1957)

Reinfection in the sensitive but immunologically deficient patient may result in cavitation due to acute necrotizing pneumonitis Such lesions could occur by either exogenous or endogenous reinfection (Schwarz and Baum 1963)

Increased resistance of mice to experimental infection has been accomplished by immunization with dead yeast cells cell wall material obtained from yeast cells and polysaccharides (Salvin 1953 Salvin and Ribi 1955 Grayston and Salvin 1956 Knight *et al* 1959)

DIAGNOSIS

In disseminated histoplasmosis peripheral blood smears and sternal bone marrow smears should be stained and examined for intracellular small (2 to 5 microns) oval bodies in the polymorphonuclear and/or mononuclear cells (Fig 22) Lymph nodes skin and mucosal lesions should be biopsied and sections studied for the intracellular parasite (Fig 23)

Blood and sternal marrow are cultured in a liquid medium brain heart infusion glucose broth Sputum and biopsy specimens should be cultured on brain heart infusion glucose blood agar at 37° C and on Sabouraud s glucose agar at room temperature Antibiotics are added to the media to prevent bacterial contamination The intradermal test

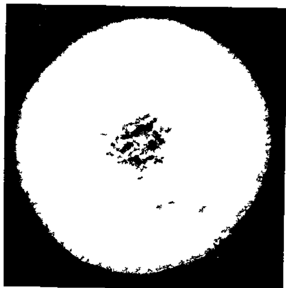


FIG 24 *Coccidioides immitis* 12 days on Sabouraud s glucose agar at room temperature



FIG 25 *Coccidioides immitis* hyphae developing characteristic arthrospore formation from Sabouraud s glucose agar at room temperature × 490

and various serologic tests referred to above may help in establishing a diagnosis The fluorescent antibody technic has been used to screen sputums for *H capsulatum* (Lynch and Plexico 1962)

TREATMENT

A variety of drugs and antibiotics have been used without success (Loosli 1957) However there have been many favorable reports concerning the effectiveness of amphotericin B in the treatment of histoplasmosis This antibiotic must be considered the drug of choice for the treatment of this disease (Newcomer *et al* 1959 Wilson 1961 Nelson *et al* 1961)

EPIDEMIOLOGY

H capsulatum exists as a saprophyte in the soil and both man and animals become infected by breathing in the highly infectious spores The disease is not transmitted from man to man or from animal to man Many epidemics have been reported involving a few or many individuals who had simultaneous exposure to the fungus in a storm cellar a cave a silo a chicken house etc (Lehan and Furcolow 1957 Wolpowitz and van EEden 1963)

COCCIDIOIDES IMMITIS

Coccidioides immitis is a spherical thick walled endospore filled organism in tissue or exudates and a fluffy white cottony fungus in culture at room temperature It produces a highly infectious disease (known as coccidioidomycosis) with an acute benign primary self limited respiratory infection, and a chronic, malignant secondary progressive *disseminated infection The secondary progressive* disease is usually referred to as coccidioidal granuloma

HISTORY

Posadas (1892) and Wernicke (1892) first described from Argentina what is known now to be *Coccidioides* in the tissue of a patient with lesions similar to mycosis fungoides Rixford (1894) first described from California a protozoanlike organism in the tissue of a patient with lesions resembling tuberculosis cutis This case and a second occurring in California were reported by Rixford and Gilchrist in 1896 Both patients were described as having primary skin lesions with subsequent lymphatic dissemination resulting in death At this time the organism was named *Coccidioides immitis* and the disease became known as coccidioidal granuloma However Ophuls and Moffitt (1900) cultured the organism proving it to be a filamentous fungus Only coccidioidal gran

uloma was known until the benign primary type of infection was described by Gifford in 1936 (San Joaquin Fever)

Cultivation

Clinical materials are cultured on Sabouraud's glucose agar at room temperature Antibiotics should be added to the medium to prevent bacterial contamination (chloramphenicol 0 05 mg /ml and cycloheximide 0 5 mg /ml)

C immitis can be grown at room temperature on all common laboratory media On Sabouraud's glucose agar at room temperature the colony develops quickly with abundant aerial mycelium which is cottony and white at first but may become powdery and buff to brown with age (Fig 24) It shows numerous arthrospores which appear as deeply staining rectangular structures 2 × 4 to 5 microns separated by clear spaces along the course of the hyphae (Fig 25) In old cultures the hyphae fragment freeing the arthrospores and the culture becomes friable and powdery Cultures should not be grown in Petri dishes because the powdery aerial growth consisting of numerous arthrospores is highly infectious The tissue phase (spherule formation) is produced in vitro only by special methods of culture (Converse and Besemer 1959) Breslau and Kubota (1964) reported temperature and CO_2 to be requirements for spherule formation in culture Brooks and Northey (1963) reported amino acids containing a ring structure (phenylalanine tyrosine and tryptophan and certain of their derivatives together with biotin) allowed conversion of mycelium to spherules in vitro Also conversion and maturation of the spherules resulted when melanin precursors and pyrocatecholamines were used

Antigenic Structure

Coccidioidin is produced in a liquid culture medium and has been used extensively as a specific skin testing antigen to detect past or present infection with *C immitis* (Smith *et al* 1948) The immunologically active material in coccidioidin is polysaccharide in nature (mannose and galactose) with small amounts of fixed nitrogen (Pappagianis *et al* 1961)

Coccidioidin also has been used as an antigen in a precipitin test and a complement fixation test to detect antibody in serum and in spinal fluid (Smith *et al* 1948 Ajello *et al* 1959 Huppert *et al* 1962) The precipitin test is positive only in recent infection and the complement fixation test is positive in chronic and disseminating disease

Immunodiffusion technics have been used to detect antibody in human serum and to analyze antigenic components of various preparations (Huppert and Bailey 1963 Rowe *et al* 1963 Rowe *et al* 1963) The spherule wall has been shown to contain immunogenic properties in experimental studies in animals (Kong *et al* 1963)

Distribution

The arid southwestern United States includes the known highly endemic areas of coccidioidomycosis The infection rate is high in the southern San Joaquin Valley in California and extends across southern Nevada southwestern Utah central and southern Arizona, southern New Mexico through western Texas along the Mexican border A high rate of infection and clinical disease occurs in the northern states of Mexico in Honduras and Venezuela The Chaco region of Bolivia Paraguay and Argentina also is an area of high endemicity

Case reports from areas other than those mentioned above usually have a history of previous residence or travel through an endemic area Sporadic cases also may have become infected from contact with contaminated materials (cotton wool etc) exported from an endemic area (Izenstark 1963 Albert and Sellers 1963)

Pathogenesis

C immitis causes an acute benign respiratory disease which is usually self limited in the white skinned race but has a tendency to develop into a progressive malignant disseminated highly fatal disease in the dark skinned races Primary infection usually takes place in the respiratory tract (rarely skin abrasions) by breathing in dust containing infectious material (Wilson *et al* 1953

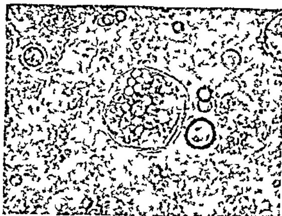


FIG 26 *Coccidioides immitis* large thick walled endospore filled spherule and smaller immature spherules in pus × 490



FIG 27 *Coccidioides immitis* section of lung showing mature and immature spherules × 490

Trimble and Doucette 1956) The infection may be subclinical or after an incubation period of 8 to 14 days the symptoms may be those of bronchial pneumonia or flu chills fever malaise anorexia cough pleurisy headache backache and night sweats About 2 to 5 per cent of such cases develop hypersensitivity which becomes evident after 5 to 14 days as typical erythema nodosum or erythema multiforme with skin lesions lasting for 1 to 4 weeks This form of the infection is not fatal and is known as San Joaquin fever Valley fever and desert rheumatism when associated with allergic manifestations

Dissemination at the time of the primary infection gives symptoms similar to those of tuberculosis Lesions may appear anywhere in the body lungs larynx lymph nodes bones joints central nervous system etc This form of the infection is highly fatal and is known as coccidioidal granuloma

Histologically the lesions are granulomata with tuberclelike formation in which the typical endosporulating spherule must be seen to distinguish them from tuberculous lesions Recently Puckett (1954) and Marshall *et al* (1955) have described the unusual occurrence of fungus hyphae accompanying the spherule stage of *C immitis* in tissues

IMMUNITY

Complement fixing antibodies and precipitins can be demonstrated in the serum of patients infected with *C immitis* (Smith *et al* 1956) The complement fixing antibodies in high titer have the same significance as in blastomycosis i e they indicate spreading infection and poor prognosis Hypersensitivity to the fungus can be demonstrated by the coccidioidin skin test A positive test usually develops in from 3 to 21 days following infection and gives a delayed tuberculinlike reaction in from 24 to 48 hours Transfer of delayed hypersensitivity to coccidioidin has been accomplished in man (Rappaport *et al* 1960) After a positive skin test to coccidioidin has been acquired the individual is immune to exogenous reinfection (Smith 1942) This fact has led to numerous investigations in which mycelial fragments arthrospores whole and disrupted spherules and endospores have been used in experimental immunization of animals (Friedman and Smith 1956 Vogel *et al* 1954 Levine *et al* 1960 1961 1962 Converse *et al* 1962 Kong *et al* 1963)

DIAGNOSIS

Pus and sputum should be examined as untreated fresh preparations under a cover glass Pleural fluid and gastric contents should be centrifuged and the sediment examined similarly Sputum and gastric contents also may be treated with antibiotics (penicillin 10 units/ml and streptomycin 40 units/ml) for an hour or two at 37 C before culturing on Sabouraud s glucose agar plus antibiotics (chloramphenicol 0 05 mg /ml and cyclo

heximide 0 5 mg/ml) In all materials *C immitis* appears as a thick walled spherule 10 to 80 microns in diameter filled with endospores (Fig 26) The resulting growth in culture is identified morphologically or after intraperitoneal injection in mice and identification of spherules typical for *C immitis* (Fig 27)

Treatment

In primary coccidioidomycosis the prognosis is excellent Treatment should be symptomatic with enforced bed rest until the temperature the sedimentation rate and the white count are normal

The secondary progressive coccidioidal granuloma has been treated successfully with amphotericin B (Klapper *et al* 1958 Littman *et al* 1958 Newcomer *et al* 1959 Perry and Kirby 1960 Wilson 1961)

Localized residual pulmonary coccidioidal lesions (coccidioidomata and cavities) require surgical management either lobectomy or segmental resection (Winn 1957)

Epidemiology

Coccidioidomycosis is a dust borne disease of the arid regions of the western and the southwestern parts of the United States Inhabitants in these areas show a high incidence of infection as demonstrated by a positive coccidioidin skin test The infection rate of susceptibles (newcomers) follows closely a seasonal variation most cases arising in the dry summer and autumn months when rainfall is lowest and dust more prevalent *C immitis* has been cultured numerous times from the soil and animals as well as man become infected There is no man to man or animal to man transmission of the disease

Control Measures

Studies carried out in conjunction with the Army Air Forces in 4 air fields in the San Joaquin Valley Calif during the period from 1941 to 1946 showed that dust control of such areas reduced infection rates from one half to two thirds in nonimmune susceptibles Paving roads and runways planting lawns and using refined oil on athletic areas proved to be effective control measures (Smith *et al* 1946)



Fig 28 *Sporotrichum schenckii* 8 days on Sabouraud s glucose agar at room temperature

SPOROTRICHUM SCHENCKII

Sporotrichum schenckii is a single-celled fusiform or round budding yeastlike fungus in the exudates or tissues of animals (rarely seen in exudates or tissues of man) and in culture on cystine-containing medium at 37 C Cultures at room temperature and on non-cystine-containing media at 37° C are leathery filamentous and usually brownish to black in color *S schenckii* frequently causes a localized lymphangitic infection and rarely a systemic infection in man

History

Schenck (1898) first cultured and described this fungus from a patient in the United States showing refractory subcutaneous abscesses Hektoen and Perkins (1900) in describing the second case named the fungus *Sporothrix schenckii* The disease was described by de Beurmann and Ramond (1903) in France and the fungus was named *S beurmannii* by Matruchot and Ramond in 1905 This species and several others described from time to time are now thought to be variants of Schenck s fungus and are usually considered as synonyms of *S schenckii*

Cultivation

Clinical materials such as scrapings from a primary chancroid lesion pus from subcutaneous nodules and sputum are cultured on Sabouraud s glucose agar at room temperature If these materials are contaminated antibiotics are added to the medium (chloramphenicol 0 05 mg/ml and cyclohexi

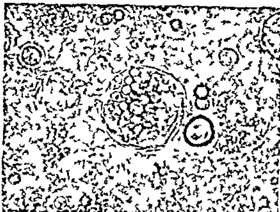


Fig 26 *Coccidioides immitis* large thick walled endospore filled spherule and smaller immature spherules in pus × 490



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Dissemination at the time of the primary infection gives symptoms similar to those of tuberculosis Lesions may appear anywhere in the body lungs larynx lymph nodes bones joints central nervous system etc This form of the infection is highly fatal and is known as coccidioidal granuloma

Histologically the lesions are granulomata with tuberclelike formation in which the typical endosporulating spherule must be seen to distinguish them from tuberculous lesions Recently Puckett (1954) and Marshall *et al* (1955) have described the unusual occurrence of fungus hyphae accompanying the spherule stage of *C immitis* in tissues

Immunity

Complement fixing antibodies and precipitins can be demonstrated in the serum of patients infected with *C immitis* (Smith *et al* 1956) The complement fixing antibodies in high titer have the same significance as in blastomycosis i e they indicate spreading infection and poor prognosis Hypersensitivity to the fungus can be demonstrated by the coccidioidin skin test A positive test usually develops in from 3 to 21 days following infection and gives a delayed tuberculinlike reaction in from 24 to 48 hours Transfer of delayed hypersensitivity to coccidioidin has been accomplished in man (Rappaport *et al* 1960) After a positive skin test to coccidioidin has been acquired the individual is immune to exogenous reinfection (Smith 1942) This fact has led to numerous investigations in which mycelial fragments arthrospores whole and disrupted spherules and endospores have been used in experimental immunization of animals (Friedman and Smith 1956 Vogel *et al* 1954 Levine *et al* 1960 1961 1962 Converse *et al* 1962 Kong *et al* 1963)

Diagnosis

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Pathogenesis

Sporotrichosis is a subacute or chronic granulomatous infection which follows introduction of the fungus by trauma The initial lesion usually appears on exposed areas particularly the extremities and develops as a single lesion (pustule ulcer abscess or chancre) which fails to heal under ordinary treatment With invasion of the regional lymphatics the characteristic picture of localized lymphangitic sporotrichosis develops ascending chronic lymphangitis with cordlike thickening of the lymph vessels and multiple subcutaneous abscesses along the course of the infected lymphatics These abscesses are gummalike and may or may not rupture spontaneously The epitrochlear and the axillary nodes are usually not enlarged and systemic reactions are rare The ulcerating nonhealing primary chancrelike lesion with lymphatic involvement suggests tularemia or syphilis This localized lymphangitic type of infection is most prevalent in the United States Single lesions about the eyes on the face the neck and on the body may not be accompanied by regional lymphangitis and create a difficult therapeutic problem unless sporotrichosis is considered (Cipollaro and Singer 1952 McGrath and Singer 1952 Singer and Muncie 1952) Other clinical manifestations have been described by Beurmann and Gougerot (1912) varying from a localized lymphangitis to a widely disseminated subcutaneous or systemic gummatous sporotrichosis An occasional disseminated case of sporotrichosis is seen in the United States (Collins 1947) Of particular concern is undiagnosed primary pulmonary sporotrichosis which may disseminate to infect various organs of the body Lesions of muscle bone and viscera (Lurie 1963) and synovitis of unknown etiology (Lynch *et al* 1963) may present problems in diagnosis Ridgeway *et al* (1962) reported 2 cases of pulmonary infection Whether or not sporotrichosis can be considered a primary pulmonary disease of any consequence must await skin testing surveys of large population groups to determine the extent of possible infection by *S schenckii*

Histologically the lesions may show only a nonspecific chronic inflammatory process or may become granulomatous with lymphocytic infiltration plasma cells giant cells and fibrosis The fusiform bodies are rarely seen in such sections or in the pus from the lesions unless the PAS stain is used (Fetter 1961) or fluorescent antisporotrichum globulin is available (Kaplan and Ivens 1960)

Immunity

Agglutinins precipitins and complement fixing antibodies can be demonstrated in the serum of patients infected with *S schenckii* (Norden 1951) A high degree of sensitivity beginning after about 5 days can be demonstrated by using heat killed saline suspensions of the fungus or a carbohydrate fraction (Gonzalez Ochoa *et al* 1947) as the skin test material A delayed tuberculin like reaction manifests itself in 24 to 48 hours

Hasenclever and Mitchell (1959) reported slight protection in mice following immunization with formalin killed yeast cells Rabbit and mouse serum containing antibodies did not demonstrate passive immunization

Diagnosis

Positive cultures are the best means of diagnosis The fusiform bodies that are found in experimentally infected animals are discovered only rarely in materials from human lesions (Fetter 1961) Pus from unruptured nodules swabs scrapings and biopsies of ulcerated lesions should be obtained and cultured both at room and incubator temperatures The characteristic colony with its typical spore formation allows identification of *S schenckii*

Treatment

Sporotrichosis responds readily to iodides administered orally over long periods Recurrences can be avoided by extending the treatment 1 or 2 months after apparent cure An occasional resistant case may respond to stilbamidine (Harrell *et al* 1954) Galiana and Conti Diaz (1963) treated 10 patients with extensive but localized sporotrichosis with thermotherapy The affected areas were heated with wet compresses and Trafuril The sustained hyperemic effect of this rubefacient was said to provide the temperature necessary for cure

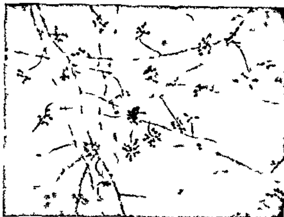


FIG 29 *Sporotrichum schenckii* delicate hyphae and conidiophores with terminal clusters of pyriform conidia from Sabouraud s glucose agar × 490

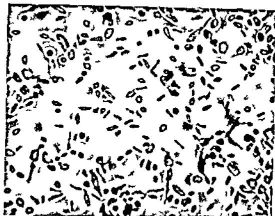


FIG 30 *Sporotrichum schenckii* fusiform cells from cystine agar culture at 37 C × 580 (Smith D T Am J Med 2 602)

mide 0 5 mg /ml) Direct examination of clinical materials will not reveal the fungus bodies unless special stains are used (PAS stain)

S schenckii can be grown at room temperature or at 37° C on all common laboratory media On Sabouraud s glucose agar at room temperature the colonies appear as small white growths lacking aerial mycelium (Fig 28) As growth increases the surface of the colony becomes folded and leathery the color may vary from white to tan or brown to black depending on the medium and the individual strain Microscopically the colony is composed of delicate branching septate hyphae 1 5 to 2 microns in diameter Pyriform conidia 2 to 4 by 2 to 6 microns in size are born at the ends of lateral branches (conidiophores) in characteristic clusters (Fig 29) In some strains these conidia are also borne directly from the hyphae On cystine agar at 37° C the growth remains soft and yeastlike and is composed of fusiform bodies (tissue phase) and short mycelial fragments (Fig 30) (Campbell 1947)

ANTIGENIC STRUCTURE

The yeast cell may be used as an antigen in the agglutination test and in the complement fixation test Heat killed saline suspensions of the yeast cells (1 1,000 packed cell volume) elicit a delayed reaction when injected intradermally Also a carbohydrate fraction from the mycelial mat or the liquid medium in which it has grown elicits a positive skin test This material will precipitate in antisporotrichum serum (Gonzalez Ochoa and Soto Figueroa 1947 Norden 1951) Using agglutination and agglutinin absorption tests Lurie (1948) was able to show a common antigen for several differently named isolates of *Sporotrichum* Kaden (1956) extracted a polysaccharide from *S schenckii* and showed it to be active in a precipitation test and by the immunodiffusion test Kunz (1959) reported successful application of the fluorescent antibody technic for culture frozen sections and smears from infected animals Also Kaplan and Ivens (1960) were able to stain *S schenckii* cells in culture and clinical materials with labeled antisporotrichum globulin

DISTRIBUTION

Sporotrichosis is world wide in distribution In the United States it is thought that endemic areas include Nebraska Wisconsin Kansas the Dakotas and Missouri Although the larger number of cases have been reported from the Mississippi Valley it is probable that numerous cases also have been recognized elsewhere but have not been reported

The fungus is widely distributed in nature and may be recovered from plants animals and a variety of contaminated objects

develops quickly as a white cottony aerial growth which becomes grayish to buff in color (Fig 31) A black pigment is usually produced in the agar on the reverse side of the colony

Microscopically single ovoid to clavate conidia 5 to 7 × 8 to 10 microns in size with truncate bases are seen on the ends of conidiophores of various lengths (Fig 32) Coremia (bundles of hyphae) may also be seen with conidia terminating the individual hyphae coming from the bundle In some strains conidia are produced laterally from the hyphae and occasionally in small clusters However the usual method of spore production is the development of single conidia from the ends of conidiophores The ascomycetous phase of *M apiospermum* is *Allescheria boydii* isolated by Boyd and Crutchfield (1921) In this culture the cleistothecia (closed perithecia) 50 to 200 microns in diameter are thin walled brownish structures containing subglobose asci in each of which are seen 8 elliptical faintly brown walled ascospores 4 to 4 5 × 7 to 7 5 microns in size

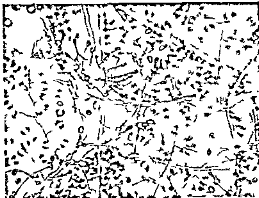


FIG 32 *Monosporium apiospermum* conidiophores terminated by a single conidium × 230 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 248 Philadelphia Saunders)

ANTIGENIC STRUCTURE

Seeliger (1956) used conidial suspensions for antigen in an agglutination test with rabbit and human serum Both culture filtrates and carbohydrate extracts were used in precipitin complement fixation and agar gel diffusion tests All of these tests were specific for the antigens used and allowed a serologic grouping of *M apiospermum* and *Madurella grisea*

DISTRIBUTION

Maduromycosis is endemic in India and the majority of the cases reported elsewhere in the world have been found in the tropics Summaries of the distribution of this infection and lists of the multiplicity of fungi which have been isolated are contained in reports by Mackinnon (1954) Lacaz and Fava Netto (1954) and Abbott (1956)

In the United States *Allescheria boydii* (*M apiospermum*) has been reported from cases of maduromycosis in Texas Massachusetts Georgia Maryland North Carolina and Pennsylvania Outside the United States *A boydii* has been reported from cases of maduromycosis in Canada the Virgin Islands Paraguay Argentina Brazil Algeria and Italy Isolations of *M apiospermum* (*A boydii*) from soil have given final proof of its saprophytic existence in nature (Ajello 1952)

Maduromycosis has also been reported in animals (Seibold 1955 Bridges 1957)

PATHOGENESIS

Maduromycosis is a chronic slowly progressive unilateral infection of the subcutaneous tissues caused by the introduction of one of several different filamentous fungi by trauma The majority of cases have occurred on the foot but occasional infections of the leg and the hand have been described Infection usually follows an injury which heals and after varying periods of time becomes noticeable by the formation of papules deep seated nodules or abscesses which rupture to form multiple draining sinuses In some instances the infection begins with swelling and pain and the subsequent development of indurated areas which become open fistulae from which drains serosanguineous fluid containing the characteristic granules With extension of the infection to the fascia muscles and bone and the development of dense fibrous tissue the foot becomes swollen club shaped and markedly deformed Osteomyelitis of the bones of the foot may cause

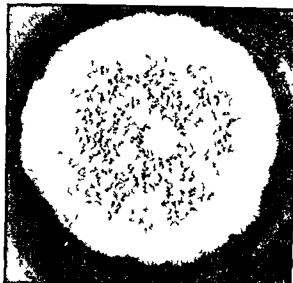


FIG 31 *Monosporium apiospermum* 15 days on Sabouraud s glucose agar at room temperature

EPIDEMIOLOGY

S schenckii has been isolated frequently from the soil Although some strains proved not to be pathogenic for laboratory animals (Howard and Orr 1963) there has been no doubt about the virulence of many soil isolates Foerster (1926) recognized sporotrichosis to be an occupational disease of horticulturists for many infections could be traced to injuries by plant thorns That the infection could assume a major role as an industrial disease was pointed out by du Toit (1942) in his report of 650 cases among miners and native workers in the gold mines of the Witwatersrand in South Africa This epidemic in the mines finally totaled 2 825 cases The fungus was isolated from the mine timbers and from the dust and infection usually followed injury by machines or rock and scratches by wire Occasional small epidemics have been reported in this country (Crevasse and Ellner 1960) Individuals in contact with infected rats dogs horses and mules may become infected

ALLESCHERIA BOYDII

Allescheria boydii is a fungus which appears as a large macroscopic grain or granule white to light yellow in color made up of wide septate hyphae in tissue or exudates from draining sinuses In culture it is a gray to light buff filamentous fungus which reproduces characteristically by single spores from the ends of conidiophores and by ascospores which are produced within cleistothecia A strain which fails to develop the cleistothecia and reproduces solely by conidia is recognized as *Monosporium apiospermum* the imperfect form of *A boydii* This fungus and many others (several genera and species) produce the disease known as maduromycosis or Madura foot

HISTORY

The discovery of maduromycosis or mycetoma in India and the early history of this type of fungous infection has been summarized by Gammel (1927) The confusion relative to etiologic agents was clarified by Chalmers and Archibald (1916) when they proposed that the disease mycetoma should be classified into two categories according to the type of fungus causing the infection (1) actinomycotic mycetoma caused by species of *Nocardia* and *Streptomyces* and (2) maduromycosis caused by the higher filamentous fungi This classification has been adopted by most investigators

M apiospermum has been isolated from numerous cases of maduromycosis in the United States and *Allescheria boydii* isolated by Boyd and Crutchfield in 1921 has been shown by Emmons (1944) to be the perfect ascomycetous stage of *M apiospermum* Other fungi reported as etiologic agents of maduromycosis in the United States include *Madurella grisea* (Neuhauser 1955) *Cephalosporium granulomatis* (Weidman and Kligman 1945) and *Phialophora jeanselmei* (Symmers and Sporer 1944 Emmons 1945 Levan 1954)

CULTIVATION

The granules obtained from draining sinuses are washed carefully in several changes of broth or sterile saline crushed between two sterile slides and the fragments cultured on Sabouraud s glucose agar at room temperature To prevent bacterial contamination chloramphenicol (0 05 mg / ml) is added to the medium On Sabouraud s glucose agar at room temperature the colony



FIG 34 *Hormodendrum pedrosoi* (*Left*) Twenty one days on Sabouraud s glucose agar at room temperature (*Center*) *Hormodendrum* type of conidiophore × 440 (*Right*) *Acrotheca* type of conidiophore × 440 (Conant N F Martin D S Smith D T Baker R D and Callaway J L Manual of Clinical Mycology p 100 Philadelphia Saunders)



FIG 35 *Hormodendrum compactum* (*Left*) Thirty eight days on Sabouraud s glucose agar at room temperature (*Right*) Conidiophore with compact spore head × 470

history of injury to bare feet it would seem that wearing shoes would be a practical control measure

HORMODENDRUM PEDROSOI

Hormodendrum pedrosoi is a small round thick walled dark brown body found in crusts exudates and tissue where it reproduces by splitting In culture it is a dark green to brown filamentous fungus which reproduces by a variety of spore forms This fungus with several others (*H compactum H dermatitidis H carrionii* and *Phialophora verrucosa*) cause the disease known as chromoblastomycosis

HISTORY

Pedroso (1911) in Brazil observed dark bodies in the tissue from a patient with verrucous skin lesions but failed to identify the fungus which he isolated Later Pedroso and Gomes (1920) reported 4 Brazilian cases including Pedroso s original patient and named the etiologic agent *Phialophora verrucosa* This identification was based on an earlier report by Lane (1915) and Medlar (1915) in which they described *P verrucosa* a fungus isolated from nodular lesions on the buttocks of an Italian in Boston in whose tissue they also had observed dark brown splitting bodies Brumpt (1922) restudied the South American fungus and named it *H pedrosoi* Terra *et al* (1922) called the disease chromoblastomycosis Carrion (1935) described *H compactum* from Puerto Rico Kano (1937) described *H* (*Hormiscium*) *dermatitidis* from a case in Japan Trejos (1954) described *H carrionii* from several isolates from patients in South Africa Venezuela and Australia

While *P verrucosa* and *H compactum* have remained constant in their morphology *H pedrosoi* has varied considerably and has presented a real problem in classification Since *H pedrosoi* has been isolated from the majority of the cases of chromoblastomycosis this has led to numerous attempts to reclassify the fungus (Conant *et al* 1954)

CULTIVATION

H pedrosoi can be cultured on all common laboratory media On Sabouraud s glucose agar the colonies are dark green to



FIG 36 *Phialophora verrucosa* (*Left*) Twenty eight days on Sabouraud s glucose agar at room temperature (*Right*) Typical conidiophore × 410



FIG 33 *Monosporium apiospermum* section from subcutaneous tissue showing granule (Smith D T Am J Med 2 602)

extensive fusion of these structures resulting in stiffness and loss of motion There is usually no systemic reaction to the infection and little if any pain

Histologically the lesions are similar to those of actinomycosis Abscess formation is prominent Granules situated in the pus may be white-yellow or black and are surrounded by a granulation tissue composed of polymorphonuclear cells plasma cells lymphocytes eosinophils and macrophages Giant cells may or may not be present The granules are rounded to lobulated structures composed of wide septate hyphae with chlamydospores around the periphery

IMMUNITY

Agglutinins have been demonstrated in the sera of several patients with maduromycosis due to *M apiospermum* In a case of chronic infection precipitins complement fixing antibodies and skin sensitivity could be demonstrated (Seeliger 1956)

DIAGNOSIS

Materials from fistulae and draining sinuses and biopsy specimens should be examined for granules These are oval lobulated 0 5 to 2 mm in diameter white to light yellow granules when *M apiospermum* is the infecting fungus Other fungi, *Madurella sp* produce black granules Microscopically the maduromycotic type of granule is composed of wide branching septate hyphae 2 to 4 microns in diameter with numerous chlamydospores (Fig 33) The actinomycotic type of granule on the other hand is composed of fine, delicate nonseptate hyphae 1 micron or less in diameter These two types of granules should be distinguished in microscopic preparations for a correct diagnosis

Granules should be washed in sterile saline or broth crushed and streaked on Sabouraud s glucose agar or beef infusion glucose agar to which antibiotics have been added to avoid bacterial contamination

TREATMENT

There is no specific therapy for maduromycosis unless amphotericin B proves to be curative Neuhauser (1955) reported promising therapeutic results with diaminodiphenylsulfone in a patient infected with *Madurella grisea* However maduromycosis is treated more effectively by surgical management supplemented by sulfonamide or antibiotic therapy to control secondary bacterial infection Early diagnosis and excision of the affected localized area should be curative

EPIDEMIOLOGY

Maduromycosis is a disease of the tropics and the subtropics with occasional cases reported in the temperate zone It is a disease of the exposed parts of the body particularly of the feet Most of the fungi belong to genera known to contain saprophytic species which may be cultured from the soil Injuries to persons going without shoes predisposes to infection Men are infected more commonly than women all races are susceptible

A boydii (*M apiospermum*) has been isolated from the spinal fluid in a case of meningitis (Benham and Georg 1948) and from the sputum in a case of chronic pulmonary infection (Creitz and Harris 1955 Tong *et al* 1958 Scharyj *et al* 1960 Travis *et al* 1961)

CONTROL MEASURES

From the nature of the infection the type of fungi involved and the almost inevitable



FIG 34 *Hormodendrum pedrosoi* (*Left*) Twenty one days on Sabouraud s glucose agar at room temperature (*Center*) *Hormodendrum* type of conidiophore × 440 (*Right*) *Acrotheca* type of conidiophore × 440 (Conant N F Martin D S Smith D T Baker R D and Callaway J L Manual of Clinical Mycology p 100 Philadelphia Saunders)



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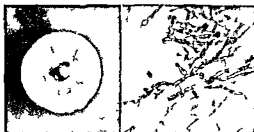


FIG 36 *Phialophora verrucosa* (*Left*) Twenty eight days on Sabouraud s glucose agar at room temperature (*Right*) Typical conidiophore × 410



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CONTROL MEASURES

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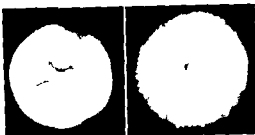


FIG 38 (*Left*) *Trichophyton (gypseum) mentagrophytes* 14 days on Sabouraud s glucose agar at room temperature (*Right*) *Trichophyton (interdigitale) metagrophytes* 14 days on Sabouraud s glucose agar at room temperature



FIG 39 Microscopic morphology of *Trichophyton mentagrophytes* (*Left*) Conidiophores producing clusters of microconidia (en grappe) × 368 (*Center*) Microconidia borne laterally on hypha (en thyrses) × 368 (*Right*) Macroconidium (fuseau) × 368

son and Gentles (1959) using this hair soil culture technic reported *A ajelloi to produce* its perfect phase and placed this in the Gymnoascaceae as *Arthroderma uncinatum* Later (1961) they described the perfect phase of *Microsporum nanum* (*Nanni..ia obtusa*) and *Trichophyton terrestre* (*Arthroderma quadrifidum*) Thus the discussions found in the early literature (Matruchot and Dassonville 1899) concerning the possible relationship of the dermatophytes to the *Gymnoascaeae* have now been substantiated

CULTIVATION

The dermatophytes may be grown on a variety of simple media but they are usually cultivated on Sabouraud s glucose agar at room temperature because the somewhat typical appearance of the colonies and the microscopic morphology developed on this medium have been used for generic and specific identification Recent studies of the exact nutritional requirements for some of the dermatophytes have resulted in a more stable taxonomy of these fungi by allowing better colony formation more consistent spore production and less variable macroscopic appearances Also cultures on hair soil medium described above have developed the ascomycetous perfect phase of some of the dermatophytes

Genus *Trichophyton* On Sabouraud s glucose agar at room temperature the colonies are granular to powdery cottony to velvety heaped wrinkled and folded with a velvety surface or heaped wrinkled and folded with a smooth and waxy surface Pigmentation of the colonies varies from delicate pink to red purple violet brown yellow and light buff

Some of the species in this genus (*T verrucosum T schoenleinii T tonsurans T megninii T equinum*) can be distinguished from each other and from all other dermatophytes by their cultural requirements Physiologic tests to determine these requirements correlated with gross colony formation on Sabouraud s glucose agar have allowed easier and more nearly accurate identification of these so-called difficult to identify dermatophytes (Ajello 1957 Georg 1957)

Microscopically microconidia are the prominent spore forms These are subspherical pyriform or clavate (1 5 to 2 × 2 to 5 microns) distributed on the sides of the hyphae (en thyrses) or produced on conidiophores in clusters (en grappe) Macroconidia are characteristic but rare and appear as long thin walled multiseptate clavate spores (4 to 6 microns in width × 10 to 50 microns in length) Raquette mycelium nodular bodies coiled hyphae and chlamydospores are also found in some species

TRICHOPHYTON (GYPSEUM) MENTAGROPHYTES (Robin) Blanchard 1896 Primary cultures may be granular to powdery and light buff to tan in color Occasional strains which produce a pink to red pigment in Sabouraud s glucose agar can be differenti

Feo and Harber 1959 Baquero *et al*, *1961)*

Epidemiology

Chromoblastomycosis is a disease of the skin of the exposed parts of the body It is *most frequent in the tropics among bare* footed agricultural laborers and others with close contact with the soil The fungi are saprophytes in nature and enter the skin by trauma The disease is not transmitted from man to man is more prevalent during adult life (30 to 50 years of age) is rarely re ported in females and shows no racial im munity

Of particular interest is the possibility of subclinical primary pulmonary infection with the fungi of chromoblastomycosis Since they occur in the soil and can be breathed into the lungs, it would be of value to know more about their possible role in systemic disease A culture of *H pedrosoi* has been isolated by bronchoscopic lavage from the bronchus of a patient with chromoblastomycosis (Baquero *et al* 1961) This culture was proved to be pathogenic by experimental in oculation into the skin of 6 humans Experi mental infection of laboratory animals has shown *H pedrosoi H compactum* and *Cladosporium trichoides* to produce brain abscess on intracerebral intravenous and intraperitoneal inoculation (Binford *et al* 1952 Duque 1961 1963) *Cladosporium trichoides* was first isolated by Binford and others (1952) from a brain abscess Since this publication many reports have been pub lished and Shimazono and others (1963) have been able to analyze 23 cases of cerebral *infection*

DERMATOPHYTES

The dermatophytes are a closely related group of imperfect keratinophilic fungi which cause specific infections of man and animals by invading only the superficial keratinized areas of the body such as the skin the hair and the nails They do not cause systemic infections and rarely invade the subcutaneous tissues In their *parasitic habitat they show* a very reduced rudimentary morphology ap pearing only as mycelial fragments in skin and nails or as mycelial fragments and arthro spores arranged inside or outside the hair However in culture on Sabouraud s glucose agar at room temperature they develop fila mentous colonies which reproduce by a variety of asexual spores characteristic of the group Four genera are now recognized *Microsporum Trichophyton Epidermophy ton* and *Keratomyces*

History

Schoenlein (1839) reported the first eti ologic agent of disease in man when he described a fungus as the cause of favus Remak (1845) named this fungus *Achorion schoenleinii* Within a few years other fungi were reported as the etiologic agents of dis ease in man Gruby (1843) described *Microsporum audouinii* and Malmsten (1845) described *Trichophyton tonsurans* as etiologic agents of ringworm of the scalp Later Sabouraud (1907) described *Epi dermophyton inguinale* from eczema mar ginatum while Castellani *(1910)* reported *Endodermophyton concentricum* from tinea imbricata or Tokelau ringworm Complete descriptions of all types of ringworm infec tion of the hair the skin and the nails as well as *the fungi which caused such lesions* were published by Sabouraud (1910) who listed 45 species of dermatophytes More than 100 species of *Trichophyton* alone are now listed in the literature These species were separated not only on the basis of dif ferences in the appearance of lesions from which they were isolated but also on differ ences observed in the gross appearance of colonies on Sabouraud s standard medium However critical studies of these derma *tophytes have reduced 2 genera and several* species to synonymy with previously de scribed forms Thus the genera *Achorion* and *Endodermophyton* have been discarded and their species have been placed in the genus *Trichophyton* This genus now con tains only 14 species while *Microsporum* contains only 7 species and *Epidermophy ton* a single species

Vanbreuseghem (1952) used the kera tinophilic property of the dermatophytes for *their isolation from the soil* By using hair bait pieces of hair on moistened soil sam ples he was able to isolate a new derma tophyte species *Kermatomyces ajelloi* Daw

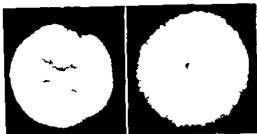


FIG 38 (*Left*) *Trichophyton* (*gypseum*) *mentagrophytes* 14 days on Sabouraud s glucose agar at room temperature (*Right*) *Trichophyton* (*interdigitale*) *metagrophytes* 14 days on Sabouraud s glucose agar at room temperature



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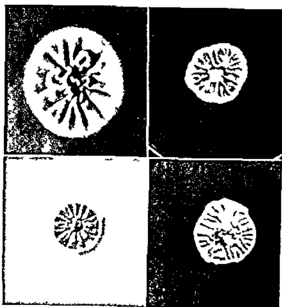


FIG 40 (*Top left*) *Trichophyton tonsurans* 35 days on Sabouraud s glucose agar at room temperature (*Top right*) *Trichophyton rubrum* 12 days on Sabouraud s glucose agar at room temperature (*Bottom left*) *Trichophyton violaceum* 19 days on Sabouraud s glucose agar at room temperature (*Bottom right*) *Trichophyton concentricum* 12 days on Sabouraud s glucose agar at room temperature



FIG 41 (*Left*) *Trichophyton schoenleinii* 21 days on Sabouraud s glucose agar at room temperature (*Right*) Favic chandeliers produced in cultures of *T schoenleinii* × 183

ated from *T rubrum* by cultivation on corn meal glucose agar in which red pigment is not produced (Bocobo and Benham 1949) Overgrowth of fluffy cottony pure white mycelium on transfer produces the inter digitale type of colony (Fig 38) Sporulation on Wort agar as well as on Sabouraud s glucose agar aids in the identification of this species Microscopically numerous subspherical microconidia tightly coiled hyphae chlamydospores raquette hyphae and nodular bodies but few macroconidia are seen (Fig 39)

This species produces an *ectothrix* infection of the hair and the interdigitale type is the common agent of athlete s foot *T mentagrophytes is a zoophilic species in that* it infects animals Laboratory infections in man have been caused by epizootics in mice rats and guinea pigs (Dolan *et al* 1958 Menges and Georg 1956)

T EQUINUM Gedoelst 1902 The colony and microscopic characteristics are similar to those of *T mentagrophytes* and many investigators believed this species to be a synonym However Georg and others (1957) found that *T equinum* has a strict requirement for nicotinic acid and feel that it can be differentiated from *T mentagrophytes* by this nutritional requirement

This species causes epizootic ringworm on horses and rarely affects man It produces an *ectothrix* type invasion of the hair

T (PURPUREUM) RUBRUM (Castellani) Sabouraud 1911 Primary cultures are cottony and pure white but later develop a velvety surface with a rose purple or reddish pigment on the back of the colony (Fig 40) Pigmentation may spread into the agar and into the surface mycelium This pigmentation is more constant when cultures are grown on corn meal glucose agar and macroconidia are more abundant when cultures are grown on blood agar base Difco Microscopically numerous clavate microconidia borne on the sides of the hyphae chlamydospores and raquette hyphae but few macroconidia are seen

This species infects the skin and the nails On the skin the slowly spreading infection persists for years and is the most difficult to treat of all the ringworm infections It is

rarely isolated from animals and probably should be considered to be anthropophilic

T (CRATERIFORME) TONSURANS Malmsten 1845 The colony is slow growing with compact whitish-cream velvety surface that becomes folded with deep crateriform depressions of yellowish color (Fig 40) Stimulation of this species by the addition of thiamine to the medium distinguishes it from *T mentagrophytes* and *T rubrum* above Macroconidia are more abundant in cultures on Wort agar Microscopically numerous clavate microconidia borne on the sides of the hyphae numerous chlamydospores hyphal swellings and raquette hyphae but rare macroconidia are seen

This species causes black-dot ringworm of the scalp in adults It produces an endothrix type invasion of the hair and is considered to be anthropophilic in that it affects man and not animals

T (ACHORION) VIOLACEUM Sabouraud 1902 The colony is slow growing heaped compact smooth waxy with irregular folds and a deep violet pigmentation (Fig 40) Better growth occurs when thiamine is added to Sabouraud s glucose agar or when this species is grown on blood agar base medium plus thiamine Microscopically only chlamydospores and hyphal swellings with no characteristic spore forms are seen

This species also causes black dot ringworm of the scalp in adults It produces an endothrix type invasion of the hair and is considered to be anthropophilic in that it affects man and not animals

T (ACHORION) SCHOENLEINII (Lebert) Langeron and Milochevitch 1930 The colony is slow growing heaped compact waxy and smooth with many irregular folds yellowish white to light brown in color (Fig 41) This species is autotrophic for vitamins and is distinguished from *T verrucosum* (below) on this basis On transfer the smooth waxy appearance changes to a velvety white Microscopically only chlamydospores hyphal swellings and the so-called favic chandeliers are seen (Fig 41)

This species is the cause of favus a severe infection of the scalp and the skin in children and adults The disease remains within family groups and in restricted localities in this country as a result of familial transfer (Loveman and Kotcher 1962) In the hair an endothrix type of invasion is seen This is an anthropophilic species

T VERRUCOSUM Georg 1950 The colony is slow growing convex disklike moist glabrous and dull yellow in color This species requires thiamine or thiamine and inositol for good growth Microscopically only chlamydospores and hyphal swellings are seen

This species affects the scalp and the skin of children but is particularly an agent of ringworm of the bearded area of the face in man It causes a severe malignant infection of the skin producing kerion a baggy indurated and inflammatory lesion It produces an ectothrix invasion of the hair and is a zoophilic species most often isolated from cattle Man becomes infected by contact with these animals

T (MICROSPORUM) FERRUGINEUM (Ota) Langeron and Milochevitch 1930 The colony is slow growing glabrous smooth and orange in color This species is autotrophic for the vitamins Microscopically only chlamydospores and hyphal swellings are seen

This species produces a microsporum invasion of the hair a sheath of spores surrounds the hair shaft The infected hairs fluoresce under Wood s light It is seldom seen in this country but causes widespread epidemics in northern Africa the Middle East and Asia It is an anthropophilic species not found on animals

T (ENDODERMOPHYTON) CONCENTRICUM Blanchard (1896) The colony is slow growing heaped deeply furrowed smooth and brownish in the center (Fig 40) On transfer the surface becomes velvety Fifty per cent of the strains studied are found to be autotrophic for the vitamins while 50 per cent required thiamine for good growth Microscopically only chlamydospores hyphal swellings and the so-called favic chandeliers are seen

This species affects only the skin producing bizarre concentric patterns of invasion It is found in Central and South America and southwestern Pacific islands It is an anthropophilic species not found on animals

The remaining species of *Trichophyton* (*T megninii T gallinae T soudanense T*

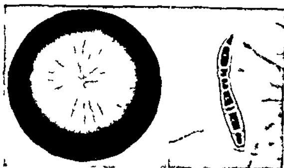


FIG 42 *Microsporum audouini* (*Left*) Twenty one days on Sabouraud s glucose agar at room temperature (*Right*) Macroconidium (fuseau) elongate imperfectly formed macroconidia are found in this species × 351

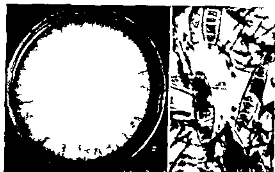


FIG 43 *Microsporum canis* (*Left*) Fourteen days on Sabouraud s glucose agar (*Right*) Well developed and numerous macroconidia are found in this species × 350

gourvilii and *T yaoundei*) occur in Europe or Africa and although the last 3 species have caused widespread epidemics in native populations in Africa they are not known to occur outside these localities *Trichophyton terrestre* Durie and Frey (1957) was isolated from soil in Australia and is not known to cause disease in man and animals Dawson and Gentles (1961) were able to culture and describe its ascomycetous stage *Arthroderma quadrifidum*

Genus *Microsporum* On Sabouraud s glucose agar at room temperature the colonies are slow growing matted and furrowed or fast growing cottony or powdery and tan to cinnamon brown in color The pigmentation in the agar may be reddish brown to orange Microscopically the macroconidia are numerous and characteristic They are large (8 to 15 microns in width × 40 to 150 microns in length) spindle shaped multicelled rough thick walled spores The microconidia (2 5 to 4 × 3 to 6 microns) scarce in primary cultures are borne singly along the hyphae or from short stalks from the hyphae

M AUDOUINII Gruby 1843 The colony is slow growing matted and velvety tan to brownish in color with yellowish or orange pigmentation in the agar (Fig 42) Yeast extracts glucose and asparagine added to medium provoke vegetative growth This species does not produce spores readily which would allow easy identification Lack of growth on polished (unfortified) rice distinguishes it from other species of dermatophytes Microscopically, the macroconidia are rare and when found in occasional isolates are bizarre in shape The microconidia are clavate (2 5 to 4 × 3 to 6 microns) borne on the hyphae or from short stalks on the hyphae Pectinate hyphae raquette mycelium chlamydospores and nodular bodies are also seen

This species is the cause of epidemic ringworm of the scalp in children The hairs are surrounded by a sheath of spores and fluoresce under Wood s light It is anthropophilic not found on animals

M CANIS Bodin 1902 The colony is fast growing with abundant cottony aerial buff tan mycelium and yellowish to orange pigmentation in the agar (Fig 43) Microscopically numerous characteristic macroconidia are produced They are large (8 to 15 × 40 to 150 microns) multicelled spindle shaped thick walled spores (Fig 43) Raquette mycelium chlamydospores and nodular bodies are also seen

This species is the cause of sporadic ringworm of the scalp in children who become infected by contact with dogs or cats The infected hairs fluoresce under the Wood s light It is a zoophilic species

M GYPSEUM (Bodin) Guiart and Grigorakis 1928 The colony is fast growing with white cottony aerial mycelium which becomes matted and powdery and cinnamon brown in color (Fig 44) Microscopically numerous macroconidia are produced They are elongate and ellipsoid (8 to 12 × 30 to 50 microns) multicelled rounded to tapering



Fig 44 *Microsporum gypseum* (*Left*) Seven days on Sabouraud s glucose agar at room temperature (*Right*) Macro conidia × 330

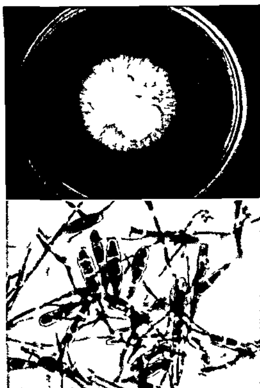


Fig 45 *Epidermophyton floccosum* (*Top*) Twelve days on Sabouraud s glu cose agar at room temperature (*Bottom*) Typically clustered macroconidia × 530

at the ends with rough thin walls (Fig 44)

This species has been isolated from soil throughout the world It infects children and adults in contact with contaminated soil or in contact with animals that have been infected from soil The hairs do not fluoresce under Wood s light Stockdale (1961) was able to culture and describe its ascomycetous stage *Nannizzia incurvata*

M NANUM Fuentes 1956 The colony is fast growing with a white floccose aerial mycelium which becomes granular in appear ance and cream colored The reverse of the colony may be tannish-orange to rose-colored Microscopically numerous macroconidia are produced They are elliptical thin walled echinulate spores (8 to 12 × 14 to 24 mi crons) with one septum or no septation A few microconidia are also found This species has been isolated from ringworm of the scalp in Cuba and Louisiana and from the skin of a patient in Alberta Canada (Brock 1961 Fuentes *et al* 1954 Carmichael and Reid 1962) It has also been isolated from infected swine (Bubash *et al* 1964) This is a zoo philic species The infected hairs do not fluoresce under Wood s light Dawson and Gentles (1961) have described the ascomy cetous stage *Nannizzia obtusa*

M DISTORTUM Di Menna and Marples 1954 The colony is fast growing the surface developing radial grooves and becoming vel vety and tan-colored Microscopically nu merous macroconidia are produced They are thick walled rough multicelled distorted in shape (4 to 14 × 3 to 40 microns) and distinctive for the species Microconidia are pear shaped borne from the sides of the hyphae

This species was isolated from ringworm of the scalp and the skin of patients in New Zealand It has been isolated from pet mon keys and dogs in the United States (Kaplan *et al* 1957) The hairs fluoresce under Wood s light This is a zoophilic species

M COOKEI Ajello 1959 The colony is fast growing with powdery yellow tan center and downy white periphery The reverse of the colony is deep purplish red in color Microscopically numerous macroconidia are produced They are elongate elliptical thick walled echinulate multiseptate (12 to 15 × 45 to 75 microns) spores Microconidia are produced

This species is a keratinophilic saprophyte that has been isolated from the soil and from

the fur of many animals that were not infected Ajello (1961) described its ascomycetous stage *Nannizzia cajetana*

M VANBREUSEGHEM Georg *et al* 1962 The colony is fast growing with a flat powdery to fluffy pink to deep rose colored surface The reverse of the colony is light yellow Microscopically numerous macroconidia are produced They are cylindrofusiform (10 × 58 to 72 microns) multicelled spores with thick rough walls Microconidia are produced

This species was isolated from a Malabar squirrel dog and human infections (Georg *et al* 1959 1962 Henington *et al* 1962) Georg and others (1962) described the ascomycetous stage *Nannizzia grubyia*

Genus *Keratomyces* On Sabouraud s glucose agar at room temperature the colony is powdery to fluffy and cream to orange tan in color Microscopically large smooth walled cylindrofusiform multiseptate macroconidia are produced Microconidia are produced in some strains This genus contains one species

K AJELLOI Vanbreuseghem 1952 The colony is fast growing flat with powdery to fluffy cream to orange tan surface The reverse of the colony may become deep purple in color Microscopically numerous macroconidia are produced They are cylindrofusiform (8 to 10 × 42 to 54 microns) multiseptate spores with thick smooth walls Microconidia are produced

This species has been isolated from soil throughout the world Many isolates have been obtained from the fur of animals but the fungus does not cause infection Dawson and Gentles (1961) described the ascomycetous stage *Arthroderma quadrifidum*

Genus *Epidermophyton* On Sabouraud s glucose agar at room temperature the colonies are velvety to powdery and greenish yellow in color Microscopically only oval to broadly clavate macroconidia are produced This genus contains one species

E FLOCCOSUM (Harz) Langeron and Milochevitch 1930 The colony develops with a central cottony white aerial mycelium which becomes powdery and greenish yellow in color (Fig 45) Microscopically the oval broadly clavate 2- to 6-celled smooth thin walled macroconidia (7 to 12 × 20 to 40 microns) are characteristic for this fungus They are produced directly from the hyphae or in typical clusters (Fig 45) No microconidia are to be found Older cultures produce many chlamydospores and raquette cells

DISTRIBUTION

The dermatophytes have a worldwide distribution However some species are found constantly in certain geographic areas and rarely in others

T schoenleinii is found in the countries bordering the Mediterranean in the Balkans and scattered throughout Europe and the Far East Cases of infection in this country usually are found in families of recent immigration *T violaceum* also is found in the Balkans Russia and southern European countries with scattered cases reported throughout Europe In the United States cases of infection by this fungus are sporadic and usually found in foreign families *T ferrugineum* is common in Manchuria and Japan and rarely found elsewhere *T concentricum* seems to have a tropical distribution it has been reported from the Pacific South America and occasionally from Central America As yet no cases have been reported from the temperate zone *T rubrum* is said to be more prevalent in subtropical areas (Mexico Central America West Indies parts of South America) In the United States more cases are reported from the southern part of the country

M audouinii is endemic in Europe (France Spain Italy Germany Austria and the Balkans) Until World War 1 the occurrence of this fungus was sporadic in England and the United States During World War II however epidemics caused by *M audouinii* in the United States and Canada have caused great concern

T tonsurans has been introduced into the United States from Mexico This fungus causes epidemic ringworm of the scalp in adults and has compounded the problem of that seen in children caused by *M audouinii*

Whether the area is rural or urban the types of infections seen and the fungi responsible for them differ greatly In rural areas man acquires his infection from contact with wild and domesticated animals The fungi

responsible for these infections cause a marked reaction on the part of the host On the other hand in urban areas man to man transmission of infection takes place and the fungi concerned with such infections cause little reaction by the host (Georg *et al* 1956 Kaplan *et al* 1958 Georg 1960)

PATHOGENESIS

The dermatophytes cause superficial infections (dermatomycoses) of the keratinized areas of the body i e skin hair and nails They do not invade the deeper tissues or internal organs of man and do not cause systemic infections in experimentally inoculated animals

The most prevalent infection is that referred to as tinea pedis (athlete s foot dermatophytosis etc) in which the toe webs are invaded by species of *Trichophyton* or *E floccosum* resulting in acute subacute or chronic infections In most instances the infection becomes noticeable as a pruritic vesiculated area between the toes with occasional spread to the rest of the foot Rupture of the vesicles and discharge of a thin serous fluid causes maceration and peeling of the tissue This may be accompanied by the appearance of fissures or cracks Unless secondary bacterial infection takes place the lesion usually persists for long periods of time as a macerated area between the toes However superimposed bacterial invasion may result in an acute inflammatory reaction with lymphangitis or lymphadenitis Occasionally certain species of *Trichophyton* cause marked inflammatory reactions and the fungus or its products sensitize the skin In such cases vesicular lesions indistinguishable from primary infections may appear elsewhere on the body particularly on the palms of the hands These lesions are considered to be allergic manifestations or dematophytids if fungi are not found in them a primary focus of infection is found elsewhere on the body and the trichophytin test is positive

Infection of the nails (tinea unguium) may accompany lesions between the toes or on the feet Usually only 2 or 3 nails are affected and these become discolored brittle opaque lusterless thickened and friable Paronychia is not common

Infection of the glabrous skin of the body (tinea glabrosa) occurs more commonly in children as a result of contact with infected animals or by autoinoculation with hairs from an infected scalp Adults may become infected by handling animals or infected children or from lesions on the nails and the feet Although a variety of lesions on the glabrous skin may be caused by dermatophytes the typical annular ringworm lesion is one with a healing scaly center and active erythematous vesiculopustular border

Ringworm of the scalp (tinea capitis) occurs in childhood and in most instances if not cured during this period heals spontaneously at puberty However a few of the dermatophytes cause lesions which tend to hold over into adult life (*T schoenleinii T violaceum T tonsurans*) Infection by *M audouinii* is acquired by contact with other infected children and usually occurs in epidemics However infection by *M canis* is acquired by contact with infected animals (cats and dogs) and is usually sporadic The appearance of the lesions depends on the infecting fungus whether a *Microsporum* or a *Trichophyton*

In microsporosis the hair is broken off a short distance from the surface of the scalp leaving grayish areas composed of hair stubs surrounded by a sheath of spores Infection by *M canis* or *M gypseum* may also cause an inflammatory reaction resulting in a boggy tumorlike mass or kerion which resembles a pyoderma In trichophytosis species of *Trichophyton* which invade the hair shaft (endothrix) cause small scattered scaly lesions with a thinning of the hair where they are broken off at the surface of the scalp leaving follicles with a black center (blackdot ringworm) Another endothrix species *T schoenleinii* causes an infection of the scalp (favus) characterized by cuplike structures (scutula) formed by the infected hair follicles Ectothrix species *T mentagrophytes* may produce acute inflammatory reactions resulting in kerion formation

Infection of the bearded region of man (tinea barbae) may be caused by *T verrucosum T mentagrophytes* or *M gypseum* and resembles closely infections due to pyogenic organisms

Since the dermatophytes invade the horny layer of the epidermis and can live and multi

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ence with those species of dermatophytes that cause an acute inflammatory response (Huppert 1962)

DIAGNOSIS

Diagnosis is made best by demonstrating the fungus in the hair the skin or the nails and by culture The Wood s light is an invaluable aid in locating and determining the extent of infection on the scalp Hairs infected with *M audouinii* and *M canis* fluoresce when this light is held close to the scalp in a semidark room Hair epilated from lesions on the scalp skin scraped from the erythematous border of lesions on the skin or obtained from the roofs of vesicles and scrapings obtained from the discolored friable areas of infected nails are examined in 10 per cent KOH Dermatophytes in the skin or the nails appear as branching fragments of hyphae (Fig 46) the genus and the species of the invading fungus can be determined only by culture In the hair the appearance of these fungi in KOH preparations allows to some extent a generic determination Species of *Microsporum* form dense spore sheaths around the hair stub with the spores crowded into a mosaic pattern (Fig 46) Species of *Trichophyton* form parallel rows of small or large spores outside the hair shaft (ectothrix microides ectothrix megaspores) or inside the hair shaft (endothrix) (Fig 46) Although the appearance of the infected hair may allow identification of the genus of dermatophyte the species can be identified only by culture

Cultures are made by inoculating Sabouraud s glucose agar slants with 2 or 3 fragments of infected material The addition of chloramphenicol (0 05 mg /ml) and cycloheximide (0 5 mg /ml) to the medium will increase greatly the percentage of positive cultures by eliminating bacterial and faster growing fungus contaminants All cultures must be maintained for at least 3 weeks at room temperature before being discarded as negative

TREATMENT

The report by Gentles (1958) that an antibiotic griseofulvin was effective in the treatment of ringworm in experimentally infected guinea pigs gave the first promise of effective systemic treatment for the dermatomycoses Continued use of this antibiotic has proved its value and it is considered the drug of choice for these infections (Gentles 1959 Russell *et al* 1960 Newcomer and Landau 1963)

EPIDEMIOLOGY

The dermatophytes include species which primarily infect animals and only incidentally infect humans (animal or zoophilic species) and species which infect humans only (human or anthropophilic species) The animal species e g *M canis* from dogs and cats or *T verrucosum* from cattle cause sporadic cases of ringworm of the scalp or glabrous skin of children or the glabrous skin of adults Usually such infections can be traced to infected animals The human species *M audouinii* causes epidemics of ringworm of the scalp of children by man to man spread of hairs infected with this fungus Such hairs are easily dislodged from the scalp and may be picked up from the backs of theater seats from the clippers in barbershops or by direct contact at play *T tonsurans* causes epidemics of ringworm of the scalp in adults

Tinea pedis (athlete s foot ringworm of the foot etc) is thought to be spread from man to man by the common use of shower baths etc in schools colleges and athletic clubs The high incidence of infection among the troops during World War II indicated again that communal life and common use of bathing facilities are important factors of spread Under such conditions the scuffed or rubbed-off infected macerated or peeling skin from the feet or between the toes serves as the source of infection *T mentagrophytes E floccosum* and *M gypseum* have been isolated from soil from contaminated shoes and/or the floor of shower stalls Man becomes infected by contact with these areas

The dermatophytes unlike all other fungi can be transmitted from man to man and from animal to man

CONTROL MEASURES

Epidemics of tinea capitis in children can be controlled by notifying public health authorities of the individual case or cases Such

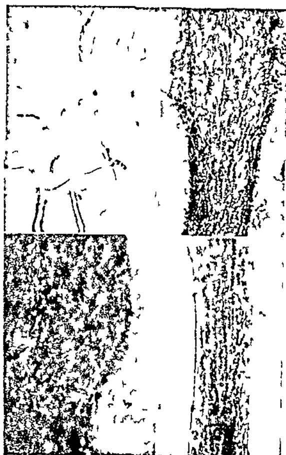


FIG 46 Potassium hydroxide preparation of skin and hair (*Top left*) *Trichophyton mentagrophytes* in skin × 415 (*Top right*) *Trichophyton* (endothrix) hair × 170 (*Bottom left*) *Microsporum* hair × 170 (*Bottom right*) *Trichophyton* hair × 170

ply only in this dead skin there are few histopathologic changes that are not those of a response to any inflammatory reaction These fungi cause erythema and edema with inflammation resulting in scaling of the stratum corneum and vesiculation Microscopically there is a marked hyperkeratosis parakeratosis acanthosis and dilatation of the vessels of the papillary layer with plasma and cellular infiltration resulting in interstitial edema

IMMUNITY

Children are susceptible to infection of the scalp and the body by dermatophytes of human or animal origin but are resistant to infection of the feet Adults on the other hand are susceptible to infection of the feet the nails and glabrous skin but are relatively resistant to infection of the scalp (Kligman and Ginsberg 1950)

Greenbaum (1924) failed to demonstrate circulating antibodies by means of the complement fixation test Marcussen (1937) by the Prausnitz Kustner technic demonstrated circulating antibodies of the urticarial type in individuals with allergic manifestations who gave an immediate wheal to intracutaneous injections of trichophytin

This immediate allergic type reaction has been found to occur not only in the atopic individual but also in individuals infected by *T rubrum* or with a history of recurrent lymphangitis (Jillson and Huppert 1949) However, immunity in humans is usually demonstrated by the cutaneous sensitivity which is established during infection and can be demonstrated by an intracutaneous injection of trichophytin The reaction is of the delayed type (24 to 48 hours) and frequently lasts 7 days A positive trichophytin test depends to some extent on the type of invading fungus and whether or not an inflammatory reaction is induced The trichophytin used to elicit the skin test in sensitive individuals contains both group specific and species specific antigens The trichophytin test may indicate either present or past infection by any one of the dermatophytes

Immunity in infected animals can be demonstrated not only by a cutaneous reaction to trichophytin but also by an acute accelerated type of lesion produced on reinoculation For varying periods of time after recovery from experimental infection the guinea pig is immune to reinfection in the previously infected site

Of particular interest is the extraction of a polysaccharide from the mycelium of *T mentagrophytes* (McNall *et al* 1961) This material was said to be antigenic for chinchillas that produced appreciable amounts of antibody The purified cell wall polysaccharide contained glucose and glucosamine

The problem of immunity in the human however resolves itself into degrees of resistance which depend on previous experi

ence with those species of dermatophytes that cause an acute inflammatory response (Huppert 1962)

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Control Measures

Epidemics of tinea capitis in children can be controlled by notifying public health authorities of the individual case or cases Such

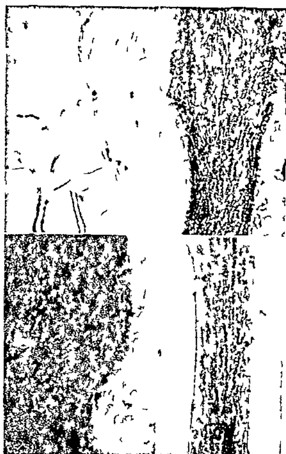


FIG 46 Potassium hydroxide preparation of skin and hair (*Top left*) *Trichophyton mentagrophytes* in skin × 415 (*Top right*) *Trichophyton* (endothrix) hair × 170 (*Bottom left*) *Microsporum* hair × 170 (*Bottom right*) *Trichophyton* hair × 170

ply only in this dead skin there are few histopathologic changes that are not those of a response to any inflammatory reaction These fungi cause erythema and edema with inflammation resulting in scaling of the stratum corneum and vesiculation Microscopically there is a marked hyperkeratosis parakeratosis acanthosis and dilatation of the vessels of the papillary layer with plasma and cellular infiltration resulting in interstitial edema

IMMUNITY

Children are susceptible to infection of the scalp and the body by dermatophytes of human or animal origin but are resistant to infection of the feet Adults on the other hand are susceptible to infection of the feet the nails and glabrous skin but are relatively resistant to infection of the scalp (Kligman and Ginsberg 1950)

Greenbaum (1924) failed to demonstrate circulating antibodies by means of the complement fixation test Marcussen (1937) by the Prausnitz Kustner technic demonstrated circulating antibodies of the urticarial type in individuals with allergic manifestations who gave an immediate wheal to intracutaneous injections of trichophytin

This immediate allergic type reaction has been found to occur not only in the atopic individual but also in individuals infected by *T rubrum* or with a history of recurrent lymphangitis (Jillson and Huppert 1949) However immunity in humans is usually demonstrated by the cutaneous sensitivity which is established during infection and can be demonstrated by an intracutaneous injection of trichophytin The reaction is of the delayed type (24 to 48 hours) and frequently lasts 7 days A positive trichophytin test depends to some extent on the type of invading fungus and whether or not an inflammatory reaction is induced The trichophytin used to elicit the skin test in sensitive individuals contains both group specific and species specific antigens The trichophytin test may indicate either present or past infection by any one of the dermatophytes

Immunity in infected animals can be demonstrated not only by a cutaneous reaction to trichophytin but also by an acute accelerated type of lesion produced on reinoculation For varying periods of time after recovery from experimental infection the guinea pig is immune to reinfection in the previously infected site

Of particular interest is the extraction of a polysaccharide from the mycelium of *T mentagrophytes* (McNall *et al* 1961) This material was said to be antigenic for chinchillas that produced appreciable amounts of antibody The purified cell wall polysaccharide contained glucose and glucosamine

The problem of immunity in the human however resolves itself into degrees of resistance which depend on previous experi

officials may then plan to screen all school children by a Wood s light and determine the extent of infection in the locality Preschool children should also be examined when an older member of the family has an infection Then examination and treatment centers may be established by the public health authorities at the schools or other convenient places

Effective control of large epidemics has been accomplished by intensive case finding and systematic follow up programs Griseofulvin allows treatment on an ambulatory basis and follow up can be assured if pills for a short time only are given at each visit to the clinic (Kirk and Ajello 1959)

Tinea pedis cannot be controlled by the use of foot baths as formerly believed Such baths (hypochlorite or hyposulfite) are not sufficiently fungistatic or fungicidal penetration of infected skin is not obtained and the time of immersion is too brief to be of value Control of infection of the feet should be directed toward individual prophylaxis adequate treatment and prevention of reinfection Foot powders (10% boric acid in talc undecylenic acid and zinc undecylenate in talc etc) tend to keep the feet dry and are of value in preventing infection Sulzberger and Kanof (1947) have reported excellent results with such powders in extensive tests Powders or ointments containing the undecylenic acid and zinc undecylenate were also found to be of value When infection is caused by a fungus known to infect animals every effort should be made to find the source and treat the animal Griseofulvin has proved to be effective for animals as well as man (Kaplan and Ajello 1959)

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38

The Principles of Epidemiology As Applied to Infectious Diseases

Epidemiology is the study of the occurrence of disease It aims to examine the varied factors that determine the development and the existence of disease or in the case of the infectious diseases the development and the existence of infection whether or not manifested by the recognizable signs and symptoms that we label disease More elaborate and complicated definitions have been developed definitions that attempt to portray some of the factors or the relationships that are involved but the foregoing seems to be the simplest and at the same time the most comprehensive

The word epidemiology is derived from the Greek words *epi* (upon) *demos* (people) and *logos* (study of) Thus from its derivation epidemiology implies the study of what falls upon the people The word epidemic evolved as a result of the early and primitive concept of disease as being caused by a mysterious force that descends on man Plagues and pestilences were originally considered to be the punishment meted out to sinful man by an offended deity Later astrologic concepts were introduced and even later climatologic ones (mal aria=bad air) Immediately prior to the advent of the bacteriologic era in the latter half of the 19th century disease was attributed to the harmful effects of poisonous miasmata and vapors that arose from decaying filth and permeated the atmosphere that man must breathe—vapors that settled on man

The term epidemiology in its original meaning thus implied the study of epidemics Through its study an attempt was made to determine the reasons for the abnormal incidence of certain infections and to explain fluctuations in the occurrence of disease It implied a special relationship to those conditions that do occur in epidemic form viz the infectious conditions even though such conditions were known to spread from person to person or from animals to persons rather than mysteriously descending on man from some hidden source on high

The purist may cling to this original concept of epidemiology but usage has broadened the term to refer to noninfectious as well as infectious conditions No one will deny the importance to be attached to the understanding of the factors underlying the occurrence of a noncommunicable disease whether of metabolic origin or due to external influences of a physical or a chemical nature One may study the epidemiology of diabetes or atherosclerosis of industrial poisonings or the effects of excessive radiation For each condition there is a multiplicity of factors that must be considered and evaluated if we are to understand why it exists

Similarly we must recognize that epidemi

ology can no longer be confined to a study of abnormal incidence or prevalence of disease Before we can understand abnormal occurrence we must understand the factors governing the normal amount An outbreak of infectious disease frequently has its origin from a single case which in turn is part of the usual number of cases that can be expected to occur Without a knowledge of why this first case occurred there can be no adequate understanding of the forces that lead to the more dramatic outbreak Knowledge of endemic factors is essential to a clear understanding of the epidemic result.

Thus like many other words in a language the term epidemiology has changed in its meaning from the narrow concept of the study of epidemics of infectious diseases to a broader concept of the occurrence of disease a study of the many diverse factors that lead in one way or another to the development of disease whether in an individual or a multitude It is in this sense that the word is used here even though the subject of this volume is diseases of bacterial origin.

As the study of the occurrence of disease the study of epidemiology must be directed to a vast array of forces that act on the animal host to produce illness Some of these forces may be biologic as for example the invasion of parasitic organisms and the reactions of the body in attempting to resist this invasion and its consequences Other forces may be physical such as climate geologic formations or radiation Still other forces may be sociologic political or even religious for the customs of man have a profound influence on his habits and thus on his exposure to conditions that may bring about disease The occurrence of many of the infectious diseases cannot be understood without considering the history of both the disease and the community for in the study of this history may be found not only some clues to why disease has occurred in the past but also some understanding of the probability of its occurrence in the future In short the science of epidemiology includes consideration of a complex array of forces that may contribute either directly or indirectly to the occurrence of disease in man

There are many possible ways in which the principles of epidemiology may be portrayed As this is a volume devoted to the bacterial and mycotic infections it seems preferable to set forth certain principles in the pattern of the development of the infectious process thus giving to the reader a more orderly approach to this subject than could be obtained from a more abstract consideration To this end one may consider the development of an infection to be conditioned by 6 related yet independent components (1) the etiologic agent, (2) the reservoir of infection (3) escape from the reservoir (4) transfer to a new host (5) entry into the new host and (6) the susceptibility of this new host.

ETIOLOGIC AGENT

Very few of the recognized bacteria are pathogenic Yet the very use of the descriptive adjective pathogenic implies certain reservations from the epidemiologic point of view for we must immediately specify for what form of life and under what conditions an organism is pathogenic

Specificity

Some organisms especially the viruses are extremely selective as to the host in which they will multiply being highly pathogenic for some species of animal and nonpathogenic for others Among the bacteria the gonococcus and the spirochete of syphilis are notable for their pathogenicity for man yet they are so harmless to other forms of animal life that no one has discovered a suitable laboratory animal in which they may be studied By way of contrast many organisms will parasitize and be pathogenic for a wide variety of hosts Notable in this regard are the salmonella many of which are found normally in other animals and attack man as an abnormal host

In many instances both the clinical picture and the epidemiologic pattern may be very different in different hosts For example *Brucella abortus* infection is highly communicable in cattle attacking the genital tract with resultant abortion and escape of the organism through the discharged fetus and the lochia In humans the infection does not attack the genital tract, plays no role in

miscarriages or abortions and is essentially noncommunicable from one person to another

Location

The mere fact that an organism can be pathogenic for the host in which it is found does not imply invariable production of disease, for it may be harmless in one anatomic situation and highly pathogenic in another The colon bacilli which normally abound in the large intestine are nonpathogenic so long as they remain in this location but their escape into the peritoneal cavity as the result of perforation of a typhoid ulcer, an intestinal wound or a ruptured appendix is followed by a severe and often fatal peritonitis Only the thickness of the intestinal wall separates man from disaster from within himself Similarly tetanus or gas gangrene spores are harmless when swallowed for they exhibit no pathogenicity so long as they are within the lumen of the intestinal tract yet if introduced into the tissues they may produce severe and often fatal illness

Type Specificity

While it is convenient to group various microorganisms on the basis of certain morphologic biologic or even pathogenic characteristics the student of epidemiology or microbiology must remember constantly that closely related organisms or even organisms that in most respects may be identical may differ markedly in their pathogenic or epidemiologic capabilities Pneumococci are indistinguishable one from the other on the basis of morphology or cultural characteristics but on biologic and immunologic bases they may be separated into more than 30 types each of which has its distinctive epidemiologic characteristics For example the age distribution of Type III pneumococcal pneumonia is different from that of Type I showing a far greater attack rate in older persons Similarly there are differences in the carrier rate of the two types Whereas the carrier rate of Type I is much higher in contacts than in the general population contacts of Type III infections do not harbor the organisms any more frequently than do persons having no such known contact If one attempts to type the pneumococci isolated from pneumonia cases during a winter season one frequently finds that one type dominates at one month a different type at other months Thus the so-called epidemic wave of pneumonia may actually be the composite of two or more separate waves each of infections of a different type of pneumococci

During the past 25 years much has been added to our knowledge of bacterial types particularly the types of typhoid bacilli and of staphylococci Not only has this provided the means for better understanding of the epidemiology of the infection but it has also provided highly valuable tools for epidemiologic investigation In the investigation of typhoid outbreaks there has long been a problem of determining which cases are part of the outbreak in question and which are the endemic cases which would have occurred in the absence of the outbreak The fact that organisms of the same phage type are isolated from several patients does not prove the interrelationship of these cases but the isolation of different types can indicate cases that are derived from different sources If a type A carrier serves a banquet we can expect that those who ate of this meal will also shed type A organisms whereas the simultaneous occurrence of typhoid in a person who did not go to the banquet presents no mystery if he is shedding type B Prior to the discovery of these type differences and the development of typing technics such cases caused much perplexity and endless confusion as attempts were made to find connections which we now know did not exist British epidemiologists have reported further use of bacteriophage typing in their search for unrecognized typhoid carriers By taking samples of sewage at various points in the collection system they have been able to identify the presence of bacteriophage for a given type trace this back to a given house connection and thus identify the carrier who was discharging both bacilli and phage into the sewage

Similarly the typing of staphylococci especially in hospital acquired infections has produced a valuable tool for epidemiologic investigation permitting the identification of cases in which there is or is not a possible

relationship and often helping to clarify the source or the mode of transmission

RESERVOIR OF INFECTION

With the discard of the old concept of spontaneous generation came acceptance of the idea that life comes from pre existing life of identical character While this latter concept has been modified slightly to recognize bacterial or viral mutation, the basic fact remains that a person develops a given infection only through invasion of organisms that came from some other source usually another person or animal harboring the organism in question A few pathogens such as certain fungi and the gas gangrene bacilli are apparently free living organisms which have the soil as their reservoir The sum total of all such possible sources is referred to as the reservoir of infection By this term is meant the natural habitat of the organism the host (or place) in which the organism normally lives and multiplies It must be distinguished carefully from the vehicle of spread which bridges the gap from source to victim Thus man is the reservoir of typhoid whereas water milk food or flies may be the vehicle of transfer jungle monkeys constitute the reservoir of yellow fever whereas mosquitoes serve as the vehicle to bring the virus from monkey to man Epidemiology is vitally concerned with the reservoir especially its character and size since the reservoir represents the ultimate source of all infections

CHARACTER OF RESERVOIR

The epidemiologic significance of a reservoir depends in large part on its character or nature Most of the bacterial infections of man have man as the reservoir and thus depend on direct or indirect transfer of organisms from one person to another The significance of a human reservoir is conditioned by the various factors that bring persons in association with one another Many other infections have as their reservoir other forms of animal life ranging from mammals through lower vertebrates to the invertebrates such as ticks and mites The significance of such reservoirs depends in part on the degree of association between man and these animals Thus little significance may be attached to a wild animal in the jungle even if it is infected with an organism pathogenic for man whereas a reservoir of infected domestic animals may constitute a serious and ever present threat Yet the wild animal reservoir may have ultimate significance as a source from which disease may escape into civilization as witnessed by the periodic escape of yellow fever virus from its natural habitat in jungle monkeys and its movement into urban areas through the woodcutters who pick up infection in the jungle Special significance may be attached to bird reservoirs inasmuch as migration may result in rapid movement of infection over vast distances and along certain paths

Travel of man has also brought about movement of disease from one area to another frequently resulting in the establishment of new foci of disease in areas previously uninfected In 1870 malaria was introduced into the island of Reunion by migrants from either Madagascar or the African mainland and became firmly established Norwegian immigrants brought leprosy to northern Minnesota where a focus of infection lasted well into the 20th century Scandinavian immigrants likewise brought the fish tapeworm *Diphylobothrium latum* which became established in the fish of many of the northern lakes Recently Neghmi described a focus in the southern half of Chile where the infection was unknown until introduced (apparently) by migrants from northern Europe

Migration of the reservoir may introduce the etiologic agent of an acute infectious disease starting an explosive and at times devastating outbreak in an area previously free of the disease In the 19th century travellers introduced measles into the Faroe Islands and the Fiji Islands with tragic consequences to populations long free of the infection In recent years a similar outbreak occurred among the Eskimos of Greenland Cholera has repeatedly spread along the lanes of international travel while influenza has moved from continent to continent as in the epidemics of 1889 and 1918 and the wave of Asian influenza in 1957 to 1958 The importance of movement of reservoirs finds expression in the international quarantine regulations some of which may be very strict

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theria has its peak about November at the same time that pertussis is near its minimum scarlet fever reaches its peak in March whereas varicella is at its maximum in December and January and measles peaks in April or even May There is less difference between the November peak of diphtheria and the September peak of poliomyelitis the spread of which has often been attributed to gastrointestinal factors due to its late summer prevalence than there is between the peaks of diphtheria and scarlet fever or those of varicella and measles

The variations in seasonal pattern often have been attributed to changes in virulence of infecting organism changes in susceptibility pattern of spread and climatic variables While it is true that such diseases as typhoid fever and poliomyelitis tend to begin and to peak earlier in the southern United States than in the North it is only in the insect borne infections that climatic factors seem to play a determining role A freeze or a killing frost can put an abrupt end to the spread of a mosquito borne infection such as dengue yellow fever or equine encephalomyelitis whereas climatologic conditions which favor the breeding of the vectors will increase the spread and therefore the size of the reservoir By contrast the winter crowding of impoverished persons will favor the transfer of lice and thus the spread of typhus if the infection is present or is introduced On the other hand the spread of malaria and therefore the size of the reservoir of active cases is definitely reduced in the cooler climates owing to the fact that the length of the period of extrinsic incubation (see p 902) varies with the temperature Thus in the cooler latitudes the number of mosquitoes that live long enough to become infectious is so small that few if any cases will develop even if new sources of infection are introduced into the community and are fed on by suitable vectors

Epidemiologic Year The fact that each disease has its own characteristic seasonal distribution means that in measuring the incidence of a disease one should not use the calendar year as a base of reference for the calendar year is a purely artificial unit of time To obtain a true measurement not only of incidence but of the magnitude of successive waves of a disease one should use the epidemiologic year This can be defined as the interval between the usual periods of minimal prevalence For example diphtheria

TABLE 1 SEASONAL INCIDENCE OF CERTAIN INFECTIONS

	MAXIMUM	MINIMUM
Diphtheria	November	August
Measles	April	September
Mumps	March	September
Pertussis	March	October
Poliomyelitis	September	March
Scarlet fever	March	August
Varicella	December to January	July to August

TABLE 2 REPORTED CASES OF MEASLES—MASSACHUSETTS 1923 1927

	1922	1923	1924	1925	1926	1927
January	1 275	3 623	2 335	1 321	6 573	719
February	2 061	3 873	3 553	2 204	6 441	855
March	2 643	3 609	4 055	2 747	5 490	1 297
April	3 603	3 863	4 197	3 846	3 776	1 406
May	4 159	4 360	3 355	3 756	3 187	1 761
June	3 198	2 985	2 392	3 094	2 724	1 734
July	1 274	1 037	939	1 037	917	1 023
August	338	271	153	338	203	253
September	268	157	143	269	70	151
October	686	660	250	1 300	120	526
November	1 167	1 096	379	3 321	161	1 221
December	2 619	1 320	674	5 583	358	2 552
Totals	23 291	26 854	22 425	28 816	30 020	13 498

in a situation in which introduction of disease holds the possibility of national disaster The stringent precautions against yellow fever that are followed by India and Pakistan are expressions of this fear of disaster as neither country has ever been invaded by yellow fever but both possess ideal conditions for its establishment and spread

SEVERITY OF INFECTION

The severity of reaction to an infection varies greatly ranging from a severe or even fatal infection through moderate to mild cases even cases that are so mild that the individual is completely unaware of the existence of the infection To a high degree these variations are probably due to differences in the number of invading organisms and in individual resistance but in certain infections the portal of entry is also a factor

From the standpoint of the individual patient, the severe infection is the most important, since it carries with it the risk of complications and of death By contrast the mild and inapparent infections are of major importance as factors in the spread of the infection Part of this is obviously due to the mobility of the infected individual for the seriously ill patient automatically goes out of circulation whether or not isolation precautions are established whereas the mild case not recognizing the true nature of the illness continues to circulate in the community, thus passing infection to other persons The fatal case is permanently removed and poses no threat Therefore from the epidemiologic point of view the mild cases are of greater importance than the severe ones

One might infer that the healthy transient carrier of diphtheria bacilli streptococci or pneumococci *would be even more infectious* than the mild case in that the former would be completely unaware of the infection whereas the latter noting at least mild signs or symptoms would be somewhat less likely to associate *with and thus infect* other persons However practical experience has shown that this may not be true and that the individual who manifests some degree of reaction to the infection is more likely to convey organisms to associates than is the person who is devoid of recognizable manifestations in spite of infection Thus Gordon *found that* the scarlet fever patient discharged from the hospital still harboring streptococci but without demonstrable signs or symptoms was less likely to infect family associates than was the patient with slight residual sinusitis or cervical gland enlargement as a result of asymptomatic pharyngeal infection Similarly Hamburger has pointed out differences in carriers of streptococci emphasizing those whom he labels as dangerous carriers Part of this distinction is in the number of organisms given off and part in the ease of escape from the patient some degree of inflammation producing a more watery and therefore more easily discharged respiratory tract secretion Whatever may be the factors involved it appears definite that all mild cases are not alike in their capacity to transmit infection (infection potential) and that for the diseases spread by way of the respiratory tract the mild case may be more important than the truly healthy carrier

SIZE OF RESERVOIR

The size of the reservoir is obviously of vital importance in understanding the occurrence of a disease for the larger the reservoir the greater the number of foci from which it may spread and therefore the greater the number of persons potentially endangered The factors governing the size of reservoirs of short lived acute infections and of long term chronic infection are quite different

Acute Infections The reservoir in this case is made up of the current cases the convalescent carriers and *the healthy carriers if* any In general therefore the size of the reservoir fluctuates according to the prevalence of the disease Three types of fluctuations are readily recognizable

SEASONAL VARIATION *Each infectious* disease has its characteristic seasonal pattern of incidence The reasons for these seasonal variations are not clear even though some degree of regularity is apparent In general *one may recognize* that infections spread by way of the respiratory tract are more common in the winter than in the summer but there are such wide variations in incidence that explanations based on temperature or crowding seem quite inadequate As noted in Table 1 based on statistics for the northern half of the temperate zone diph

resulting split wave In such cases seen frequently in measles a moderately high incidence occurs in each of two successive epidemiologic years instead of a much higher incidence confined to a single year In such cases the second part of the splitwave commonly will occur much earlier in the epidemiologic year than is usual because of the relatively large number of cases that continue to occur during the season of minimal prevalence and therefore the unusually large number of foci from which new infections will develop

Diseases such as diphtheria scarlet fever or meningococcal meningitis which are less highly communicable manifest similar though less dramatic year to year variations The first two show peaks every 3 to 5 years whereas meningococcal infections recur at irregular intervals In some measure these variations can be explained on the same basis as measles (changes in the number of susceptibles) but for the irregular and unpredictable waves of meningococcal infection there is as yet no more satisfactory explanation than for those of poliomyelitis or influenza Many hypotheses have been advanced but none seems entirely satisfactory

LONG TERM VARIATION Aside from the seasonal variation and the short term periodicity of diseases one may recognize various long term cycles for which no satisfactory explanation has been advanced Thus diphtheria which had not been a major problem in the first half of the 19th century rose to a peak about the 1870 s alarming in both its severity and the number of cases For example the deaths and death rates in Massachusetts showed a peak in 1875 to 1879 followed by a progressive decline during the rest of the 19th and the first part of the 20th century It is hard to attribute this to human factors of control for the rate had declined sharply before the discovery of antitoxin showed no sharper decline after its introduction and was at a comparatively low level before active immunization was introduced in the early 1920 s Similarly scarlet fever for which no effective therapeutic or prophylactic agents were developed prior to the sulfonamides showed striking declines in both severity and mortality It is impossible to attribute these changes to any man made influences especially when so far as can be determined from old records both diseases had showed alternating periods of high and low incidence and severe and mild infections during previous centuries

Chronic Infections The reservoir of long term infectious diseases including those in which there is a chronic or permanent carrier condition is largely dependent on the past history of the disease and consists of two parts the active and the convalescent cases and the recovered but still infected persons The number of the former varies with the current incidence which may be high or low depending on prevailing circumstances However the number of recovered but still infected individuals is dependent on the past history of the disease For example a state which has had a high typhoid incidence during the previous 25 to 50 years obviously will have a larger number of chronic carriers than a state in which the typhoid incidence has been low Similarly the reservoir of carriers will decline automatically and ultimately will disappear as the disease is kept under control A simple example will suffice to illustrate this phenomenon

Let us assume that in 1913 state X had 5 000 persons who recovered from typhoid fever and that 2 per cent of these became permanent carriers This means that 100 carriers were produced in that year On the assumption that the average age of typhoid cases was 20 years and that at that age a person had a life expectancy of 50 more years we could think of these 100 carriers as dying in 1963 not from typhoid fever but from heart disease cancer and other conditions that are important causes of deaths at age 70 If the typhoid rate in state X had been reduced progressively to the level of only 50 persons who recovered from typhoid in 1963 2 per cent of whom became carriers there would have been only 1 carrier produced in that year to take the place of the 100 who had died Thus the size of the reservoir was reduced by 99 not because of any changes in community sanitation that were put into effect in that year but simply because the reservoir was dying out faster than it was being replaced Thus year after year as the incidence of typhoid is kept at its present low rate the reservoir becomes smaller because

TABLE 3 REPORTED CASES OF MEASLES IN MASSACHUSETTS BY CALENDAR AND EPIDEMIOLOGIC YEARS

Calendar Year	Cases	Change From Previous Year	Epidemiologic Year*	Cases	Change From Previous Year
1923	26 854		1922 23	28 361	
1924	22 425	−16%	1923 24	24 212	−15%
1925	28 816	+28%	1924 25	19 789	−18%
1926	30 020	+4%	1925 26	39 784	+101%
1927	13 498	−55%	1926 27	9 757	−75%

* September 1 through August 31

is usually at its minimum in July or August Hence the diphtheria wave should be studied from August 1 of one year to August 1 of the succeeding year By contrast poliomyelitis is at its minimum in March or April so that the epidemiologic year may be considered to run from April 1 to the same date in the next calendar year

When studied on the basis of the epidemiologic rather than the calendar year the true variations in the incidence of a disease become much more appparent Table 2 shows the reported cases of measles in Massachusetts for the years 1922 to 1927 In Table 3 these data are totaled on the basis of calendar years and on epidemiologic years running from September 1 through August 31 September being the usual month of minimum incidence On the basis of calendar year totals the incidence increased 28 per cent from 1924 to 1925 and another 4 per cent from 1925 to 1926 Yet when the totals from the epidemiologic years are compared it is noted that there was actually an 18 per cent decrease from 1923 24 to 1924 25 and an increase of 101 per cent from 1924 25 to 1925 26 Similarly the decline in the subsequent years was 55 per cent on the basis of the calendar year but 75 per cent on the basis of the epidemiologic year It is apparent that the epidemiologic year totals give a much more accurate picture of the relative magnitude of successive seasonal waves of the disease Calendar years are useful for non scientific purposes but for serious study of the occurrence of infectious disease they are often highly misleading and should be replaced by the epidemiologic year

SHORT TERM CYCLES It is common knowledge that the incidence of many infectious diseases varies greatly from year to year Infections such as measles, poliomyelitis or influenza show striking variations, being almost absent in certain years and breaking out in explosive waves in other years There is a certain degree of regularity with respect to the recurrent waves of measles which occur every 2 to 4 years in urban areas and at longer and more variable intervals in less crowded communities No comparable regularity in the spacing of waves of influenza or poliomyelitis is apparent In all of these conditions however a severe wave of infection with a large number of cases is followed the next year by a very low incidence even a virtual absence Similarly the longer the interval between peak incidence the more severe the ensuing wave These variations are commonly and probably correctly explained in large part on the basis of numbers of susceptibles The incidence of measles declines when the number of susceptibles in the community declines to the point at which few of the persons being currently exposed are susceptible to the infection In other words the epidemic wave ends when the number of susceptibles is too small to keep the disease spreading Consequently the year following a year of abnormally high incidence will have very few cases because of the small number of susceptibles The longer the interval between epidemic waves the greater the number of susceptibles who will have been added to the community with the result that when the infection is introduced again a larger number of cases will develop Because of the late beginning of a wave sometimes the number of susceptibles is not sufficiently reduced before the seasonal decline and the disease may carry over into the next season with a

sels but finding no portal of escape The likelihood of spread of an infection is thus conditioned in large part by the portal of escape and as a consequence by the anatomic the physiologic and the cultural factors relevant to a particular portal

Respiratory Escape Unquestionably the respiratory tract provides the most important portal of escape and the one that is most difficult to control The respiratory tract secretions are driven from the body by the propulsive force of chest muscles and diaphragm varying from the quiet exhalation of a person at rest to the forceful ejection that accompanies a cough or a sneeze As the respiratory passages are normally moist and bathed in a secretion slight departures from normal as a result of inapparent infection pass unnoticed Furthermore the nose and the mouth serve as portals of both escape and entry Normal association of persons brings the portals of escape of one person only a few feet or inches from the portals of entry of another These characteristics not only facilitate the spread of infection from the respiratory portal but to all intents and purposes preclude control measures such as have been so effective in dealing with diseases caused by organisms which escape by other portals

Intestinal Escape By contrast escape through the gastrointestinal portal lends itself more readily to control Whereas the respiratory tract is a dead end the gastrointestinal tract is a tube through the body The normal discharge is through defecation an intermittent physiologic function usually performed once or twice a day but often less frequently In most societies cultural patterns attach an element of modesty and therefore privacy to defecation Therefore gastrointestinal escape can be limited to certain locations where the material can be collected and treated to destroy pathogens Thus while the absolute numbers of microorganisms that escape may possibly be greater than those that escape from the respiratory passages the associated anatomic physiologic and cultural characteristics are such that gastrointestinal escape constitutes a less dangerous and more readily controllable mechanism as shown by man s success in controlling such diseases as typhoid or cholera as contrasted with failure to control the common cold or in the absence of immunization diphtheria or smallpox

Genitourinary Escape Somewhat similar considerations determine the epidemiologic significance of escape by the genitourinary portals except in those situations in which spread may occur through direct physical contact as in the spread of venereal diseases On the other hand it is essential to remember that urination occurs more frequently than does defecation that in a rural population it is commonly performed wherever the person is working and thus may result in contamination of the working environment A striking example of this is seen in the spread of bilharzia the schistosome eggs escaping with the urine and contaminating the irrigation ditches or rice paddies where the workers hands arms and feet come in contact with water containing cercariae after snail passage

Abnormal Portals In certain cases organisms may escape through artificial portals which because of their character may be less important than the normal portals The scarlet fever patient who develops a draining otitis media is discharging streptococci with the ear drainage but is far less likely to spread infection than the patient with a residual sinusitis or pharyngeal involvement The ear discharge is propelled only by the force of gravity or pressure within the middle ear the discharge is an obvious abnormality and can be caught on an ear plug of cotton and thus destroyed Thus even though the number of organisms in the discharge from an abscess may be greater than in a similar quantity of respiratory secretion the physiologic factors are so entirely different that the epidemiologic significance is far less

Percutaneous escape is similarly effected in the absence of an anatomic portal However this necessitates the intervention of an outside force which brings about a break in the skin to release the organism In diseases such as malaria yellow fever typhus or bubonic plague the blood sucking insect effects the escape from the reservoir and later brings about both the transfer of the organism and its entry into the new host Man plays the same role in the case of hepatitis malaria or syphilis spread through blood

of the impact of the degenerative diseases which are killing the carriers produced in former years In other words if we made no further improvements in our community sanitation measures, which have been so effective in the control of typhoid we would nevertheless see a steady decline in the typhoid rate as the number of sources (the reservoir) declined as a result of the death of carriers from conditions entirely unrelated to their carrier state Thus so long as we so much as hold the line in sanitation, typhoid will become less and less serious and will ultimately disappear simply because of the *disappearance of the reservoir of chronic carriers* In the United States we have already reached the point at which the principal factor in the year to year decline in the typhoid rate is not the current *improvements in sanitation* but deaths of carriers who were produced in former years when the disease was far more prevalent

The epidemiologic influence of a declining reservoir is seen also in tuberculosis The tuberculosis death rate has been falling steadily from a rate of over 350/100 000 population in 1875 in a typical state (Massachusetts) to a rate of only 6 4 in 1960 This has meant that there have been fewer active cases to spread infection to others The drop in the percentage of the population at a given age who are tuberculin positive (infected) gives striking evidence of the reduction in the spread of infection Concurrent with the decline in mortality and infection rates has been a *shift into the older age groups* due *not* to an increase in cases in the elderly but rather to a greater drop in the rates in the young adults than in those over 50 In 1960 in the United States 81 per cent of the total tuberculosis deaths were in persons 45 or older as contrasted with 27 per cent in 1915 41 per cent were in persons 65 or older These older persons are those who lived through an era when there was more tuberculosis than at present they represent the survivors of the cohorts who were infected in earlier years In essence this means that the principal reservoir of tuberculosis is in *older persons* Many of these are dying of tuberculosis Doubtless an even larger number of persons with clinically inactive infections are dying of other conditions before they break down with the active and communicable form of the disease The result is a rapid disappearance of the reservoir of tuberculosis as infected and therefore potentially infectious persons are dying faster than they are being replaced It is popular (and clinically flattering) to attribute the current sharp decline in tuberculosis mortality to the use of current chemotherapeutic agents notably streptomycin isoniazid and para aminosalicylic acid Unquestionably these drugs have prolonged many lives and have rendered many patients noninfectious but the disappearance of the reservoir is probably a more significant factor *than* is any form of therapy As in the case of typhoid fever we are currently reaping the benefit of programs instituted by earlier generations in that the reservoirs are pre*dominantly older persons who lived through* an era when the disease was more common and infection more prevalent and are now reaching the age when mortality rates are high and therefore are dying off more rapidly than they are being replaced

ESCAPE FROM RESERVOIR

The mere existence of a reservoir of infection of a certain size does not mean that there is an equivalent number of potential sources of infection To spread to other persons the microorganism must find a portal of escape from the reservoir lacking such there can be no spread Actually there are numerous situations in which there is a large reservoir *but little or no transfer* The classic example of this is in the case of trichinosis an infection in which the trichinal larvae are encysted within the muscle fibers Although human infection rates in the United States have been estimated to be as high as 15 per cent none of these persons is a source of potential spread unless man turns to cannibalism The only significant part of the total reservoir is that in swine, since eating infected *pork* permits release of the trichinal larvae which mature in the intestinal tract of the eater where they mate and produce a new generation of larvae which migrate to the muscles Most of the reservoir of syphilis is likewise of no significance as a source of infection inasmuch as the spirochetes in late syphilis are deeply seated damaging the walls of the blood ves

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the secondary levels has changed with the development of consolidated schools which bring together in an urban environment children who otherwise would have been relatively isolated in small or even one room rural schools The school bus which has brought the children to the consolidated school has contributed further to direct respiratory association just as within an urban area crowded buses and subway trains bring the individual into all but actual facial contact with innumerable persons any one or several of whom may be sources of infection

Aside from these community factors that condition the degree of direct respiratory spread we must recognize factors within the home During the past half century there have been striking shifts in the age distribution of certain infections such as scarlet fever that cannot be explained on the basis of immunization or improvement in community sanitation This phenomenon was also seen in diphtheria before the advent of active immunization To a certain degree this shift of disease into the older age groups may be correlated with better housing but the change in family size is probably of greater significance In earlier days when families were large the younger preschool children who had relatively few extra familial contacts were exposed to the infections brought into the home by older siblings With the decline in family size and the increase in the number of one or two child families (the children in the latter case being closer in age) the preschool group has been less exposed with a resultant shift of infections into the school age group In other words the social pattern of family size has resulted in a somewhat increased isolation of the highly susceptible infant and preschool child with a resultant delay in direct respiratory transfer of infection

In quite a different way cultural factors affect the degree of direct physical contact that results in the spread of venereal infections The ultimate factor here is biologic only in the sense of response to a fundamental biologic urge However cultural patterns which regulate reactions to this instinct have developed The pattern of sexual behavior is a creation of the human mind and therefore has developed somewhat differently in different societies Trial marriages and mistresses have become a part of certain cultures and are defended on the grounds of deterrents to divorce which may be looked on as a greater social evil Some cultures have tolerated and even licensed prostitution Even in a culture such as has developed in the United States the attitude toward promiscuity varies greatly among different social groups as manifested by the extent of extramarital relations and community attitudes toward prostitution Thus while in our culture we may think of sexual contacts (direct physical spread) from the standpoint of morality we cannot ignore the existence of social and anthropologic factors both in our culture and that of other nations that have influenced the extent of such contacts and thus the incidence of venereal disease

Indirect Transfer

The probability of indirect spread depends on two factors the ability of the microorganism to survive for a suitable period of time outside its host and the existence of a suitable vehicle by means of which it can be transferred to a new host

Resistance of Organisms Microorganisms differ greatly in their sensitivity to the effect of potentially damaging external forces of the environment such as acidity drying heat radiation and the action of various chemicals Typhoid bacilli can survive in a more acid medium than can dysentery bacilli and both are more resistant to acid than is the cholera vibrio As a consequence of these differences milk plays a definite role in the spread of typhoid a more minor one in the spread of dysentery and none at all in the spread of cholera The vegetative form of the *Entamoeba histolytica* is so delicate that it dies quickly outside the human body and even if it should be swallowed would be destroyed by the normal acidity of the stomach By contrast the amebic cysts are sufficiently resistant to acidity drying and temperature that they spread readily through water or food and if swallowed are not destroyed in the stomach Organisms which develop protective spores or cyst walls are obviously more resistant than those which

transfusion The lack of a normal portal of escape and the nature of the force that effects escape through artificial portals definitely facilitates control of these diseases

Duration of Escape Diseases differ greatly in the period of time over which the organisms escape from the reservoir In some infections the period is very short as in the case of influenza or measles which are infectious for only a few days Other diseases such as diphtheria or scarlet fever remain communicable for several weeks whereas still others such as leprosy may continue to be infectious throughout life In general the period of communicability of the viral infections is shorter than that of bacterial or mycotic infections

The infection potential (degree of communicability) of a disease varies inversely with the length of the period of communicability A disease with as short a period of communicability as measles or influenza (only a few days) must spread readily to other persons if the infection is to survive in nature Were its communicability as slight as that of leprosy it would never have appeared as a disease or to put it another way would have died out long ago Conversely leprosy has such a low infection potential that its period of organism escape must be long Were its period of communicability as short as that of measles or diphtheria it also would have died out centuries ago or would never have got started The inverse relationship between infection potential and duration of escape of the organism is a biologic necessity

TRANSFER OF INFECTION

In order to establish a new infection pathogenic organisms that have escaped from the reservoir must be transferred to a new host This transfer may be either direct or indirect

Direct Transfer

Direct transfer implies such close association between source and new host that there has been a virtually instantaneous passage In some diseases this may be through actual physical contact as in the spread of venereal disease Direct respiratory spread does not necessarily imply actual contact as in kissing but it does require such close physical approximation that the organisms breathed out by the source are immediately inhaled by the victim The fact that the respiratory portals serve for both escape and entry obviously facilitates direct transfer as normal human relations frequently bring the nose and the mouth of one person only a few feet (or even inches) from the nose and the mouth of another

While anatomic and physiologic considerations are important factors in direct spread sociologic economic and cultural factors are of equal if not greater ultimate significance as it is these that bring people into close physical contact or association The degree of direct respiratory spread is a function of crowding i e the frequency with which persons come close enough to each other to permit one person to inhale the organisms that the other has just exhaled In the crowded low economic section of a large city the individual obviously will have close respiratory association with more people (more potential sources of infection) than will the person who lives in the less congested high income area This association finds expression in the different age distribution of respiratory infections in the two groups In the former situation a higher proportion of the children of a given age will have had measles or (in the days prior to active immunization) be Schick negative than in the latter situation The crowding that is seen in institutions of various types or in military barracks similarly favors direct respiratory spread

Until recent years similar differences were observed in comparisons of rural and urban groups the former showing much less evidence of direct respiratory spread owing to relative isolation Today this difference is less noticeable and often even absent due to changes brought about by developments in transportation Improved highways and economic factors which have made automobiles available to families previously served only by a horse or possessing no independent transportation have markedly reduced the isolation of the rural dwellers bringing them frequently into more crowded urban areas As a result of better transportation the system of education at the elementary and

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Until recent years similar differences were observed in comparisons of rural and urban groups the former showing much less evidence of direct respiratory spread owing to relative isolation Today this difference is less noticeable and often even absent due to changes brought about by developments in transportation Improved highways and economic factors which have made automobiles available to families previously served only by a horse or possessing no independent transportation have markedly reduced the isolation of the rural dwellers bringing them frequently into more crowded urban areas As a result of better transportation the system of education at the elementary and

planation for the well recognized fact that a carrier may handle food for long periods without producing recognizable disease Similar considerations enter into the chance of spread of other diseases through food

AIR The advent of the bacteriologic era gave support to the concept of the spread of disease by air but this idea was discarded as a result of the studies of Fluegge who postulated that bacteria are not suspended in the air but fall to earth in discrete droplets unless inhaled through direct transfer Thus air was considered to play no role in the spread of infection until some years later when the studies of Wells showed that various microorganisms can remain suspended in air in the form of droplet nuclei These may be as small as single organisms covered with a thin film of moisture held on the surface by capillary attraction greater than the pressure of vaporization On this basis it became clear that air may serve as a potential vehicle for spread of infection as shown by the occurrence of certain infections explicable only on the basis of air transfer

The fact that under certain conditions air can serve as a vehicle for transfer of infection does not mean that it does so to an important degree There is a vast difference between a bacteriologic possibility and an epidemiologic probability Experience has shown that air may serve as a vehicle under special circumstances as in operating rooms nurseries and possibly other hospital situations in which a patient is essentially immobilized under conditions which eliminate or minimize the chances of direct respiratory transfer Under such conditions the few organisms that are in the air and fall into a wound or are inhaled may be of significance because of the high level of susceptibility However under conditions of normal community life the number of organisms in the inhaled air is so small as contrasted with those acquired through direct respiratory association that they have no significance in persons who through these daily contacts have of necessity a fairly high level of resistance It is thus only in certain diseases (such as Q fever) or under special circumstances that air transfer is of appreciable epidemiologic significance

FOMITES At one time various inanimate objects such as bedding books clothing doorknobs money or toilet seats were looked on as important vehicles for the spread of infection In the absence of known exposure to a recognized source of infection it was easy to speculate as to contact with some object with which a person had had direct or indirect contact which resulted in contamination The later development of knowledge of carriers and subclinical infections resulted in a de-emphasis of the role of fomites with resultant reduced attention to the elaborate rituals of disinfection that characterized public health practice at the turn of the century While doubtless there are situations in which certain fomites may convey infection their role is usually very minor and far smaller than that commonly attributed to them by the general public which characteristically clings quite tenaciously to basic ideas acquired in childhood

SOIL For certain infections the earth may serve as a vehicle or rather as a harborage for organisms deposited on it Anthrax and tetanus bacilli may persist in the ground for many years protected against adverse environmental factors by their heavy and resistant spore walls The character of the soil and its cover of vegetation are major factors in the epidemiology of hookworm disease in that they affect the survival of the larvae between hatching of the egg and effective contact with human skin If the soil is dry sandy and not protected by vegetation the larvae will die quickly through exposure to the sun whereas larvae on moist soil protected by adequate vegetation may remain alive for long periods of time and hence have a better chance of reaching a new victim The probability that the soil will be contaminated and that bare skin will come in contact with it depends of course on the culture and the economy of the people viz their habits of defecation and the extent to which shoes are worn or agricultural patterns that bring the hands in contact with the soil

INSECT SPREAD Whereas ticks and mites (which are arthropods and not insects) may serve as reservoirs of infection insects can serve only as animate vehicles of spread As such they serve as vectors a term once used to refer to all types of vehicles both animate and inanimate but now by common usage

ing preparation are also those that are not cooked subsequently or are not cooked to the point of adequate heating of the interior of the food mass

The likelihood that food will cause infectious disease on a given occasion depends on a series of independent variables

1 The probability that the food handler is infected

2 The number of viable organisms discharged This is extremely variable ranging from very few on certain days to enormous numbers on other days This variation is dependent to a high degree on the speed of passage through the intestine If a typhoid carrier is constipated the bacilli discharged from the gall bladder may be largely overgrown by the colon bacilli before they are discharged with the feces whereas the number of viable bacilli may be large if the carrier is suffering from a diarrhea or even has a fairly rapid passage of intestinal content

3 Contamination of the hands at the time of defecation This again is highly variable ranging from gross fecal soiling to minimal bacterial contamination

4 Thoroughness of hand cleansing after contamination likewise a varying quantity depending on the habits of the individual Even at best and using thorough scrubbing far beyond normal usage there can be no assurance of complete removal of all contaminating pathogens

5 Duration of time and type of activities between defecation and handling of food Intestinal pathogens on the skin tend to die fairly quickly even in the absence of further cleansing procedures In addition the habits of the individual may lead to repeated hand washing during the interval between defecation and handling of food Thus a housewife who is a typhoid carrier and normally defecates after breakfast usually will not handle food until several hours later during which interval some of the organisms on the hands will have died and others been removed by the numerous handwashings incidental to her housework Thus she will have few organisms left to transfer to food in contrast with the larger number that would have been present had she set aside her housework in favor of preparation of food for a noon time social gathering

6 Type of food handled whether to be cooked or eaten without further heating adequate to destroy the pathogens

7 Quantity eaten

8 Susceptibility of those eating the food

Very obviously, there is a vast difference between an occasion when a carrier discharges many organisms, heavily contaminates the hands, washes carelessly promptly handles food which will not be cooked and will be eaten in large quantity by susceptible persons as contrasted with an occasion when the carrier gives off few viable organisms barely soils the hands washes thoroughly delays several hours before handling food which will be cooked or if not cooked will be eaten in small quantity or by people who are resistant In the former case an outbreak of food borne disease will result in the latter the number of organisms even if ingested by a susceptible person may not be enough to produce illness but will evoke an antibody response which adds to the person s resistance Thus it is a common observation that typhoid carriers may handle food over long periods of time without causing recognizable disease and yet on a given day will cause a serious outbreak On such occasions the victims are not members of the carrier s immediate family but rather are persons who do not regularly eat food prepared by the carrier Over the years the carrier has latently immunized (see p 903) her immediate family through repeated small doses of organisms which were adequate to stimulate antibody production but never adequate to cause disease To cause infections new susceptibles must be brought within her sphere of influence

From the foregoing it is apparent that the transmission of disease depends on a series of related yet independent variables or probabilities to each of which we might in theory attach a mathematical value The probability of simultaneous occurrence of two or more independent variables is the product rather than the sum of the individual probabilities In this case there are at least 8 variables the individual probabilities of which must be multiplied by each other to determine the likelihood of disease from this person on a given occasion From the magnitude of this product is to be found the ex

infectious By contrast if the period is long the majority of the mosquitoes that acquire the infection will not live long enough to become infectious Thus in the first situation *the disease spreads readily and becomes* established among the people once it has been introduced In the latter situation it fails to become established and will even die out in spite of repeated reintroduction simply because too few mosquitoes live long enough to become infectious A few cases of in digenous malaria may develop as a few mos quitoes succeed in living through a long period of extrinsic incubation but the num ber that survive is so small that over a period of time the infection dies out even in the absence of effective mosquito control or eradication procedures Thus the inci dence and the resulting prevalence of malaria infection in a given area are dependent in part on geographic factors which govern the availability of a suitable vector in part on climatologic factors governing the duration of extrinsic incubation and in part on social or economic factors that determine the extent of contact between man and mosquitoes

ENTRY OF INFECTION

To a high degree the entry of an organ ism is the converse of its escape depending on the portal of entry and the numerous ana tomic physiologic and sociologic factors as *sociated with that portal* As in the case of the portal of escape there is a general rela tionship between the portal of entry and the subsequent clinical manifestations those or ganisms entering through the respiratory portals producing respiratory tract involve ment, whereas those which are ingested affect the gastrointestinal tract However this gen eralization while useful in the epidemiologic investigation of a disease of undetermined character cannot be carried too far for or ganisms that are ingested may attack the throat (milk borne scarlet fever or diphthe ria) while certain viruses that apparently are spread by way of the respiratory tract appear to be responsible for cases or outbreaks of nausea vomiting and diarrhea

It is important to remember also that sev eral and at times abnormal portals of entry can exist and that the character and the sever ity of a disease may be altered by the portal of entry Plague bacilli entering through a flea bite produce lymph node involvement (bubonic plague) whereas those which are inhaled produce pneumonia (pneumonic *plague) Similarly inhalation of relatively* small numbers of the rickettsiae of Q fever results in illness whereas the drinking of milk containing large numbers of rickettsiae from infected cattle appears to produce an asymp tomatic infection detectable only by antibody formation Careless use of rectal thermom eters or tubes within a nursery has resulted in spread of gonococcal infection which has manifested itself through an asymptomatic proctitis complicated by gonococcal arthritis The classic example of modification through abnormal portal was the old but now dis carded practice of smallpox inoculation which through introduction of the virus into the skin, produced a milder form of the disease than resulted from respiratory entry In the pre Jennerian days this had been the basis of smallpox control

SUSCEPTIBILITY

Just as the likelihood that an individual attacked by a specific microorganism will be come infected depends on his degree of re sistance so also the incidence of a particular disease within a community is dependent on the extent to which the people in general are resistant The individual may acquire resist ance *from an attack of the disease from a* series of repeated small doses of organisms (latent immunization) or through artificial immunization The resistance of the com munity depends on the number of persons who through one or another of these mech anisms have developed an adequate level of personal resistance

For certain diseases the baby possesses a high level of resistance at birth because of the presence of antibodies that have passed through the placenta from the mother to the fetus This passive resistance is dependent on the resistance of the mother and is lacking if the mother has not herself acquired an active resistance It persists longer than does the passive resistance from injection of anti bodies falling off at about 3 to 4 months and in most cases disappearing by the end of six months For certain infections however the maternal antibody even if present in sig

limited to refer only to insects The female insect even though infected does not transmit her infection to the next generation through the egg This means that the insect to pass infectious disease to man must make two contacts first to acquire the organism from a reservoir and then to transmit it to a new host This in turn requires that the insect after acquiring the infection must live long enough to become infectious a factor that varies in importance according to the mode of transfer

The simplest type of transfer and one that does not necessitate direct personal contact is that of mechanical surface contamination as illustrated by the fly that soils its feet while walking over feces containing typhoid or dysentery bacilli and then rubs these organisms onto food by walking over it In other instances there may be internal contamination as the fly feeding on the excreta ingests pathogens which pass inertly through its gut and are deposited on food through defecation In either situation the importance of flies in the spread of the infection will depend in part on the number of flies available in part on the number of persons discharging the pathogens in their feces and in part on the practices with respect to disposal of excreta and food protection Under certain circumstances flies can be and have been factors of major importance under other circumstances they have been of no importance

The biting insect by contrast must have actual physical contact with the reservoir as well as with the new victim This depends to a high degree on man s habits and customs such as type of housing (including use of screens) type of clothing and his presence in places where and at times when the insect is biting Transfer in this case may be by simple contamination of the mouth parts as in the case of African sleeping sickness (trypanosomiasis) or through systemic infection of the insect as in the case of the rat flea in bubonic plague or the louse in epidemic typhus In these latter two the insect is also suffering from and usually dies from its infection Thus as Zinsser has stated the insect (if it could have a point of view) might look on man as a vector of disease

Whereas transmission by flies, fleas and lice involves a relatively simple mechanism that by mosquitoes is more complicated in that it introduces a new variable namely the period of extrinsic incubation By this is meant the length of time between the intake of pathogens and the ability of the mosquito to transmit them to a new host, or in other words the time for the organisms to migrate from the mosquito stomach through the coelomic cavity to the salivary glands, whence they are injected into the wound whenever the mosquito bites During this period of migration there may be changes in the pathogen as in the case of malaria but a change of this sort does not occur in yellow fever A comparable phenomenon is seen in rabies Before a dog that has been bitten by a rabid animal can transmit the infection the virus must travel from the wound to the salivary glands a migration that may require several weeks or even months The virus does not appear in the saliva until a few days prior to the onset of symptoms Thus even though the dog is incubating rabies one can assume that it was not in an infectious condition if it is still alive and well at the end of 10 to 14 days the usual period of observation after biting

The length of the period of extrinsic incubation depends on several environmental or biologic variables As the mosquito is a cold blooded animal the speed of its metabolic processes varies with the external temperature Thus the period of extrinsic incubation is short in regions of high temperature and longer in colder climates It varies also with humidity species of anopheles and strain of plasmodium i e there is a short period with *Plasmodium falciparum* in the *Anopheles gambiae* of equatorial Africa a longer period with *Plasmodium vivax* in *Anopheles quadrimaculatus* in a temperate zone such as the United States and an even longer period for *Plasmodium malariae* wherever it occurs

The duration of the period of extrinsic incubation is in many respects the most important factor in determining the prevalence and the incidence of a disease like malaria in an area where a suitable vector exists If the period is short a high proportion of mosquitoes that pick up the sexual forms of the plasmodium by feeding on infected humans will survive long enough to have the organism reach the salivary gland and thus become

sons) is introduced into a completely susceptible community It will produce 2 cases each of whom in turn will produce 2 more or 4 Each of these will produce 2 more with the resultant development of 8 cases Therefore the spread of the infection within the community will be by geometric progression as follows 1—2—4—8—16—32—64—128— Under such circumstances the infection would increase rapidly ultimately declining only when most of the persons exposed are those who have already been infected and recovered (resistant)

If by contrast this same infection were introduced into a community in which half the people were resistant (either thru artificial or latent immunization or prior attack) the one case exposing 2 others one of whom was already resistant would produce only 1 new infection This person in turn exposing 2 others only 1 of whom was susceptible would also produce only 1 new infection The result would be a series of sporadic infections 1—1—1—1—1—1—1—1— rather than the explosive outbreak Thus the incidence of the disease has been reduced not simply to half but out of all proportion to the fraction of the population that is resistant If the infection potential had been 3 this same result would have been achieved if two thirds of the population were resistant if it had been 4 only if three fourths were protected Thus the greater the infection potential (communicability) the greater must be the fraction of the population which is resistant if the disease is to be reduced to mere sporadic proportions

Fortunately experience with artificial active immunization bears out the foregoing theoretical reasoning In such diseases as smallpox or diphtheria control of which is based on active immunization the incidence of the disease has been reduced out of all proportion to the fractions of the population that have been actively protected Those who are not protected and are therefore susceptible have a vicarious protection through the immunization of a large enough fraction of the total population so that mere chance reduces the likelihood that an infected person will come in effective contact with a susceptible

Obviously in the foregoing it has been assumed that the person who is resistant does not develop either the carrier state or a mild inapparent infection as a result of a resistance high enough to protect against overt disease but not adequate to prevent a low grade but communicable infection If we are dealing with a viral disease in which the carrier state either does not exist or is uncommon this theoretical objection would not apply However even in the bacterial infections there is no evidence that to any significant degree the inapparent infections in immunized or partially resistant persons occur with sufficient frequency to upset the generalization of reduction of disease out of all proportion to the fraction of the population immunized or resistant In the early days of diphtheria immunization many persons worried lest by immunizing a fraction of the population the number of unrecognizable cases and carriers would be so increased as to jeopardize the nonimmunized segment Fortunately studies have shown a striking decline in the carrier rate coincident with an increase in immunization

The Epidemic Curve

The foregoing principle of the effect of immunizing a fraction of the population is a significant factor in the rise and fall of the epidemic curve for certain diseases It has been pointed out already that measles occurs in cyclic waves usually spaced 2 or 3 years apart in sizable communities having frequent contact with other communities but more widely and irregularly spaced in smaller communities especially if somewhat removed from the flow of people In the former a few cases of measles are occurring constantly or isolated cases are frequently introduced but in neither situation does the disease increase to epidemic proportions until the number of susceptibles is large enough so that there is a high probability that the infecting cases will come in contact with susceptibles As the epidemic curve rises the number attacked and therefore converted to nonsusceptibles increases until ultimately the proportion of immunes is so large that it offsets the number of susceptibles At this point the peak of the epidemic is reached and the curve declines with resultant fading out of the disease as the ratio of immunes to susceptibles becomes greater and greater The disease disappears not because there are no

nificant quantity, fails to pass the placental barrier, with the result that the newborn infant is highly susceptible Notable in this respect is pertussis which may develop within the first month of life due to lack of transmitted antibody whereas measles is rarely seen before 6 months of age simply because most women by the age of childbirth have had measles and their resultant antibody can pass to the fetus in utero

After the disappearance of this passive resistance the baby starts to develop his own active resistance depending on exposure to infection For most bacterial infections this is by a process of latent immunization as a result of repeated exposure to small doses of organisms The dose may never be large enough to produce symptoms or the resultant infection may be so mild as to escape recognition but in either case the body is stimulated to produce antibodies Over a period of time as a result of these repeated low grade and usually unrecognized infections the body develops a level of resistance adequate to protect against a dose which earlier might have produced serious or even fatal infection It has thus been latently immunized a process of active immunization

The rate at which this process of latent immunization progresses depends on the likelihood of exposure to the infection in question If the infection is highly prevalent as was diphtheria in former days resistance may be acquired fairly early in life a high proportion of 5 to 10 year old children being resistant even in the absence of known infection By contrast there is so little diphtheria in most communities today that in the absence of artificial immunization almost all children and a high proportion of adults are susceptible The rate of latent immunization likewise depends on the degree of contact between persons It is higher in urban than in rural areas although as pointed out earlier the changes in transportation and the development of consolidated schools have resulted in a less pronounced difference between urban and rural susceptibility than existed formerly

Although the pattern of development of resistance in the individual is very different in the bacterial and the viral infections the community patterns are quite similar, in that the proportion of persons of a given age group who are resistant increases with age In the bacterial infections this has been produced by a combination of active cases and latent immunization in the viral infections by active cases In either case the older age group will show a higher proportion of resistant persons Unfortunately however the resistance to the bacterial infections is less likely to persist than is that to viruses, and is thus highly dependent on continuing exposure to the forces of latent immunization Thus in former days when diphtheria and typhoid fever were prevalent the individual who had been latently protected through repeated small doses retained his resistance as a result of continuing exposures Today, with the very low prevalence of these diseases the likelihood of exposure (latent immunizing force) is so low that the individual usually loses whatever resistance he had acquired through latent or artificial immunization The result has been a noticeable shift in the age distribution of these diseases a significantly higher proportion of the cases occurring at a much older age than in former years By contrast the person who has had such viral infections as measles smallpox chickenpox or yellow fever tends to retain this resistance even in the absence of continuing exposure possibly due to the intracellular character of viral infections The classic studies of Panum in the Faroe Islands from which measles had been absent for 65 years until it was reintroduced in 1846 showed clearly that, even in the absence of continuing exposure the resistance to measles may persist for 65 years or longer whereas persons who had never had or been exposed to measles are susceptible regardless of age

Effect of Partial Community Protection

Although the incidence of a given disease depends on the proportion of a population that is susceptible the mathematical relationship is not one of simple proportion Both in theory and in experience it is found that the incidence of a disease is decreased out of all proportion to the fraction of the population that is resistant

Let us assume that a single case of a disease with an infection potential of 2 (each case on the average infects 2 or more per

tions in which shorter periods warrant study and, accordingly rates may be expressed on the basis of months or even weeks Here again there is the necessity of stating clearly the unit of time on the basis of which the rate is calculated Many serious errors have crept into the literature because of failure to include a precise statement in the expression of rates

Rates may be calculated either by use of a formula or even better by establishing a proportion and solving for the unknown In this latter case one asks himself how many events might be expected to occur in a standard unit of population if a certain number of these events occurred in a certain population Thus if 5 000 deaths occurred in a population of 500 000 persons in a year then one could expect that 10 deaths would have occurred for every 1 000 population for the same year The formulas that are frequently used are calculated from such proportions and are useful shortcuts only if used correctly Too often the user transposes numerator and denominator or unit population and actual population errors that cannot be made if the rates are calculated on the basis of proportions

The rates most commonly used in epidemiology are as follows

Crude Mortality Rate

This is the number of deaths per 1 000 population per year It can be calculated on the basis of the formula

$$MR = \frac{\text{Number of deaths}}{\text{Actual population}} \times 1\,000$$

In the example cited above this would be

$$MR = \frac{5\,000}{500\,000} \times 1\,000$$

$$= \frac{5\,000}{500} = \frac{50}{5}$$

$$= 10 \text{ or } 10 \text{ deaths/1 000 population/year}$$

If determined through establishing a proportion and solving for the unknown this would read

$$\frac{5\,000}{500\,000} = \frac{y}{1\,000}$$

which is mathematical shorthand for the question If 5 000 deaths occurred in a population of 500 000 persons then how many would be expected in a population of 1 000 persons for the same year? Solving this proportion,

$$500\,000\,y = 5\,000 \times 1\,000$$

$$y = \frac{5\,000}{500\,000} \times 1\,000$$

$$y = \frac{5\,000}{500} = 10$$

It will be noted that the right hand side of the second equation is the same as the formula

The crude mortality rate is probably the most accurate of all the commonly used rates There is official recording of deaths except in frontier or primitive society The only important inaccuracy in this rate is the population which is the mid year estimate for the year in question For censal years this figure is relatively exact but for intercensal years the population must be estimated This is usually on the basis of changes between censal years (10 years apart) on the assumption that trends during this interval will be continued unabated in the years following the latest census This is not always a correct assumption, with the result that the *denominator of the crude death rate may be* in error and is certainly less accurate than the numerator

Specific Death Rates

Whereas the crude death rate includes all deaths specific death rates include only a specific group of deaths having in common one or more specific characteristics Thus one may specify a great variety of attributes that separate certain deaths from all other deaths Among the attributes that are commonly specified are sex age color and cause Thus one may calculate the death rates of males or of females death rates at various ages or for various causes of death At other times one may combine attributes such as deaths of white women age 40 to 44 due to cancer of the stomach in this case a color sex age-cause specific rate In calculating specific rates care should be taken to use as a population only those persons whose deaths could have been included in the numerator Thus in the foregoing situation the

longer any susceptibles, but because the number of these has been reduced to the point that the infecting cases will come in contact with immunes rather than susceptibles Stocks has suggested that in measles at least some of the noninfected may have developed a transient resistance which must disappear before the next wave of the disease can develop but evidence to substantiate this hypothesis is lacking Nor is such a hypothesis necessary to explain the decline of the outbreak for the mere probability of contact with an immune or a susceptible adequately explains the shape of the epidemic curve It is not necessary to assume that everyone in a community will have developed some measure of resistance before an infection ceases to spread or is reduced to mere sporadic cases

The longer the interval between epidemics the greater the number of susceptibles who will have been added to the community and therefore the greater the magnitude of the epidemic wave Conversely a severe outbreak as of measles or poliomyelitis will be followed by a period of unusually low incidence because of the extremely small number of susceptibles to whom the disease may be spread

In some infections such as poliomyelitis the number of inapparent infections is so large as compared with those that are recognizable that the reduction in the number of susceptibles is out of proportion to the apparent incidence of recent infections There is strong evidence to suggest that fairly widespread poliomyelitis infection may occur in the absence of any large number of recognizable cases especially during the so called off season If in reality this does occur a community may be left with too few susceptibles to permit the development of an outbreak whereas a neighboring community that has not been invaded recently by the virus has so many susceptibles that it experiences a sizable outbreak Observations of waves of poliomyelitis have shown repeatedly the existence of islandlike areas which are spared the effects of an outbreak of considerable magnitude in neighboring communities The hypothesis of community resistance through the recent occurrence of large numbers of unrecognized infections provides an attractive explanation for which there is considerable supporting evidence Here also, the fundamental phenomenon is the probability of effective contact between a source and a susceptible person

RATES COMMONLY USED IN EPIDEMIOLOGY

Since epidemiology concerns itself with the mass phenomena of disease occurrence there is an obvious need for units of measurement by which to describe the amount of disease (or infection) that occurs The mere enumeration of events might suffice in certain circumscribed situations but would be grossly inadequate in comparing the impact of disease on populations differing in size or character To make valid comparisons one must resort to rates which basically are ratios between the number of events that occur and the population at risk Since in theory the event in question might happen to every member of the population in question a rate expresses a ratio between the number of events that occur and the number that theoretically might occur

To permit comparison rates are calculated as the number of events per unit of population By convention the unit to which events are calculated varies somewhat according to the rate in question though rates calculated to unconventional units are not mathematically wrong However enough variation exists that it becomes absolutely necessary in all cases to state the size and the character of the population with respect to which events are calculated For example a death rate for cancer of the prostate obviously should be expressed as the number of deaths from cancer of the prostate per unit number of males Of course lacking exact data on the proportion of males in the population one might express this as the number of prostate cancer deaths per total population To make the rate clear to other persons it would be essential to state whether the base of reference is the total population or only the male population Similarly it would be essential to define the span of time, for obviously the rate for a year would be very different than the rate for a month When a span of time is involved the convention is to use the year as a unit but there are many situa

subject to gross error is almost always too low and must be taken with a high degree of reservation Special caution must be used in comparing rates of different communities for while in theory the quality of medical care may be equal vast differences are known to exist in the extent to which illness is brought to medical attention and in the completeness of reporting of diagnosed cases

Attack Rate

The term attack rate is frequently used to portray a morbidity or case rate within a circumscribed population having a known exposure to infection It thus differs from the usual morbidity rate which is based on total population of a community only a fraction of whom would be actually exposed Attack rates usually expressed in percentages are often used to describe the frequency of an infection within a family or an institutional group in which a primary case has occurred It thus describes the frequency of secondary infections among the known contacts The term secondary attack rate is commonly used in such situations

Incidence vs Prevalence

Just as the terms mortality rate and fatality rate are frequently confused and misused owing to superficial similarity of ideas so also unfortunate errors are commonly encountered through misunderstanding of the difference between the incidence and the prevalence of a disease The word incidence derived from the Latin word *incidere* meaning to fall upon or to strike refers to the number of new cases that develop in other words how frequently a particular infection strikes the people of a community Thus the incidence rate is the same as the morbidity or case rate and the terms may be used interchangeably It is usually expressed as so many newly reported cases per hundred thousand population per year though different units of population or spans of time may be used if clearly indicated

By contrast the prevalence rate of a disease expresses the amount of a particular disease that exists or prevails within a given population at a particular moment of time It includes all cases that exist at a certain time regardless of how long these cases have existed Thus while an incidence rate for syphilis would be based on the number of persons who acquired this infection during a year the prevalence rate would express the proportion of the population who have syphilis regardless of how long the disease has existed Some of these will be new cases but obviously most will be old cases of varying duration

The difference between incidence and prevalence is an important distinction in epidemiologic thought Unfortunately too many persons have used the terms as though they were synonymous with resulting confusion and even mistaken conclusions To say that the prevalence rate of syphilis in a certain population is 5 per cent means that 5 per cent of these people are infected with syphilis some of them recently infected but others infected many years previously It does not mean that in a given year 5 per cent of the people can be expected to acquire syphilis yet exactly such conclusions have been drawn and published by persons unfamiliar with the correct meanings of the terms incidence and prevalence

Whereas incidence rates are commonly expressed in number of cases per hundred thousand population per year prevalence rates are most commonly expressed in percentages They are used extensively in the study of chronic diseases noninfectious as well as infectious whereas incidence rates are more commonly used in the study of acute short term infections In the latter the incidence is higher than the prevalence since it includes all cases developing over a span of time In chronic infections the prevalence is greater since it includes old cases regardless of their duration while the incidence rate includes only those acquired or recognized during a given span of time

SUMMARY

As the science concerned with the occurrence of disease (or infection) epidemiology must consider a multiplicity of factors that in one way or another bring about contact and interaction between pathogenic microorganisms and the host on which these organisms act In the strict sense of the word it

rate would be calculated as so many deaths per unit number of white females age 40 to 44 in the population in which the deaths occurred Most commonly, cause specific rates are calculated to a base of 100 000 population of the same attributes as the deaths in question There is less uniformity as to rates specific only as to sex, color or age hence the importance of stating clearly the units involved in the rate

In general specific rates are less accurate than are crude rates owing to inaccuracies with respect to the specific attributes The exact age of the deceased is often an approximation while errors or inaccuracies as to the cause are well known Undoubtedly as diagnostic procedures improve cause specific rates become more nearly accurate yet diagnosis is still far from perfect Furthermore statistically according to the International Classification of Causes of Death one may die from only one cause Thus the factor underlying a death may be overlooked in preference to the condition that brought about the ultimate demise A patient who in the delirium of typhoid fever leaps from a tenth story hospital window will be classified as a suicide rather than a typhoid death thus obscuring the full impact of typhoid on the community in question

Fatality Rate (Case Fatality Rate or Ratio)

This rate usually expressed in percentages measures the proportion of persons ill with a particular disease who die from that disease *It must be distinguished from the* mortality rate which measures the number *of deaths from a given disease per unit of* population The difference is illustrated best in the following tabulation of 3 hypothetical communities of equal size

	A	B	C
Population	100 000	100 000	100 000
Cases of typhoid	10	100	1 000
Deaths from typhoid	1	10	100
TF mortality rate	1/100 000	10/100 000	100/100 000
TF fatality rate	10%	10%	10%

In the foregoing the fatality rate is the same in all 3 communities viz 10 per cent for 1 of every 10 cases died However the mortality rate ranges from 1 per 100 000 population in community A to 100 per 100 000 population in community C Thus the fatality rate describes the disease whereas the mortality rate describes the community In serious diseases the fatality rate is high even though the disease may be so rare that the mortality rate is low e g the fatality rate of human rabies a rare infection is 100 per cent while the mortality rate of measles an almost universal disease is much higher than that of rabies even though the fatality rate is low (less than 1%)

The fatality rate expressed by the formula

$$FR = \frac{\text{Deaths}}{\text{Cases}} \times 100$$

obviously is a ratio between deaths and cases The official data as to deaths from a certain cause are relatively accurate whereas the data as to number of cases are highly inexact as they depend on the extent to which the cases are recorded Cases may go unrecorded because the family fails to seek medical care a correct diagnosis is not made or the physician fails to report the case A high proportion of measles cases are never brought to medical attention while in an infection such as poliomyelitis the mild subclinical infections are so lacking in distinctive pathognomonic signs or symptoms that an accurate diagnosis is often impossible without time-consuming and frequently expensive laboratory studies Thus the fatality rate based on the ratio between deaths and reported cases of the disease frequently gives a false and exaggerated impression of the *true severity of the illness*

Morbidity Rate (Case Rate)

This rate usually expressed as the number of reported cases per 100 000 population per year measures the extent to which new cases occur or are recognized within a community Since as pointed out above many cases are never brought to official attention and therefore are not recorded this rate is

subject to gross error is almost always too low and must be taken with a high degree of reservation Special caution must be used in comparing rates of different communities for while in theory the quality of medical care may be equal vast differences are known to exist in the extent to which illness is brought to medical attention and in the completeness of reporting of diagnosed cases

Attack Rate

The term attack rate is frequently used to portray a morbidity or case rate within a circumscribed population having a known exposure to infection It thus differs from the usual morbidity rate which is based on total population of a community only a fraction of whom would be actually exposed Attack rates usually expressed in percentages are often used to describe the frequency of an infection within a family or an institutional group in which a primary case has occurred It thus describes the frequency of secondary infections among the known contacts The term secondary attack rate is commonly used in such situations

Incidence vs Prevalence

Just as the terms mortality rate and fatality rate are frequently confused and misused owing to superficial similarity of ideas so also unfortunate errors are commonly encountered through misunderstanding of the difference between the incidence and the prevalence of a disease The word incidence derived from the Latin word *incidere* meaning to fall upon or to strike refers to the number of new cases that develop in other words how frequently a particular infection strikes the people of a community Thus the incidence rate is the same as the morbidity or case rate and the terms may be used interchangeably It is usually expressed as so many newly reported cases per hundred thousand population per year though different units of population or spans of time may be used if clearly indicated

By contrast the prevalence rate of a disease expresses the amount of a particular disease that exists or prevails within a given population at a particular moment of time It includes all cases that exist at a certain time regardless of how long these cases have existed Thus while an incidence rate for syphilis would be based on the number of persons who acquired this infection during a year the prevalence rate would express the proportion of the population who have syphilis regardless of how long the disease has existed Some of these will be new cases but obviously most will be old cases of varying duration

The difference between incidence and prevalence is an important distinction in epidemiologic thought Unfortunately too many persons have used the terms as though they were synonymous with resulting confusion and even mistaken conclusions To say that the prevalence rate of syphilis in a certain population is 5 per cent means that 5 per cent of these people are infected with syphilis some of them recently infected but others infected many years previously It does not mean that in a given year 5 per cent of the people can be expected to acquire syphilis yet exactly such conclusions have been drawn and published by persons unfamiliar with the correct meanings of the terms incidence and prevalence

Whereas incidence rates are commonly expressed in number of cases per hundred thousand population per year prevalence rates are most commonly expressed in percentages They are used extensively in the study of chronic diseases noninfectious as well as infectious whereas incidence rates are more commonly used in the study of acute short term infections In the latter the incidence is higher than the prevalence since it includes all cases developing over a span of time In chronic infections the prevalence is greater since it includes old cases regardless of their duration while the incidence rate includes only those acquired or recognized during a given span of time

SUMMARY

As the science concerned with the occurrence of disease (or infection) epidemiology must consider a multiplicity of factors that in one way or another bring about contact and interaction between pathogenic microorganisms and the host on which these organisms act In the strict sense of the word it

refers to man and has been so treated in this chapter, but from the scientific standpoint one might equally consider comparable factors governing the occurrence of disease in animals (epizootology)

Since epidemiology deals with man it must take into consideration various factors of his biologic processes his social behavior and the environment in which he lives The relative importance of these varies with different diseases but there is probably none in which a single factor is operative On the contrary disease or infection may be thought of as the result of interaction of a complex array of variables—an interaction system far more complex than that which any chemist ever concocted within his test tubes or flasks Some of these forces are physical and can be measured with a fair degree of precision others are biologic and therefore less easily quantitated whereas some of the most important lie in the realm of the behavioral sciences and are even less subject to exact measurement Within the laboratory of the physical scientist interaction systems may be devised which measure the force of a single variable by keeping all other factors constant In the biologic laboratory this is less possible because of the complexity of various biologic forces and the difficulty or the impossibility of keeping any of these constant Even less exact must be the measurements with respect to man who enjoys a free living existence in a physical and social environment of enormous complexity and variability

The epidemiologist attempts to unravel this complex skein of interacting forces to measure as best he can the magnitude of these forces and their relative importance The problem is complicated by the fact that many of these forces are constantly changing in magnitude as for example the size of the reservoir of infection Therefore the epidemiologist must recognize that he is dealing with a dynamic rather than a static problem and that as a consequence the epidemiologic characteristics of a given disease may change from one period of time to another and from one place to another No greater mistake can be made by the student of epidemiology than to adopt a static rather than a dynamic point of view

In this context it should be emphasized that the epidemiologist deals with probabilities especially when he considers infectious disease Infection is the resultant of a chain or series of independent yet related events or circumstances which must take place in proper sequence to produce a new infection Just as a chain is no stronger than its weakest link so is the chain of infection dependent on the strength of its separate components In this case strength is measured in probabilities and the final effect is the product of these separate probabilities He who seeks to control the development of a given infection or disease examines this chain to determine the strength of its various links and concentrates his attention on the weakest Thus epidemiology not only serves to further the understanding of disease but is also the foundation on which control measures are based

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39

The Prevention of Infections

Many of the most significant preventive measures for the control of microbial infections arose from empiric observations made centuries before precise knowledge of the etiology and the means of transmission of any such infection was established Thus the inoculation of smallpox crusts into the nostrils of infants to produce a mild form of smallpox which led to immunity against the severe natural disease apparently dates back to the pre Christian era in India and China Similarly isolation of persons *suffering from dreaded* diseases such as leprosy goes back into early history and reflects an awareness of the concept of contagion The development of quarantine in Venice in the 14th century is another example of the fact that people had long ago become aware of the dissemination from man to man of what we now call infectious diseases Food as a source of such illness also appears to have been recognized in ancient times A widely accepted example is the series of restrictions in ancient Hebrew law against eating shellfish or pork—sanitary regulations so to speak that had a logical basis in the light of our present knowledge of the risk of consuming such foods without adequate control of their procurement and handling

PRINCIPLES OF CONTROL

A knowledge of the sources of the infectious agents that cause disease in man as well as of the patterns of their transmission provides a logical basis for outlining the possible means of control Smith (1941) vividly describes a number of the basic defences against infection and introduces a number of concepts which are important to understanding mechanisms of control The etiologic agents of human infectious disease must in all cases establish residence in some reservoir which may be man himself (e g diphtheria) another animal (e g brucellosis) or the environment (e g histoplasmosis) If the organism is to survive in a living reservoir host it must be capable of *establishing a parasitic* relationship without producing severe illness or death in the reservoir host From this reservoir the agent must be transmitted to a susceptible human host by a mechanism which is characteristic for each agent When disease patterns are viewed in this framework three general areas of control become apparent neutralization of the disease reservoir interruption of the chain of transmission from reservoir to man (or man to man)

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of the organism by deleterious factors (drying sunlight heat etc) before it contacts a susceptible host Dilution may also shift the ratio of clinical to subclinical infections because there appears to be a direct relationship in many infections between the infecting dose and the proportion of those infected who develop clinically apparent disease A specialized instance of this approach is the inspection of animals in slaughterhouses where the removal of obviously infected animals from processing greatly reduces the incidence or intensity of contamination in the output of the plant

FILTRATION The mechanical process of filtration is widely used to remove microorganisms from water air and pharmaceutical preparations and when properly controlled is highly effective The use of ground filtration galleries provided a significant reduction in the incidence of waterborne disease in the period before water chlorination was generally adopted (see Fig 1) On the other hand the common surgical face mask is a relatively ineffective filter unless great care is taken in its design

Physical and Chemical Methods Heat and sunlight are perhaps the oldest forms of disinfection or sterilization known The cooking of foods or their preservation by drying in sunlight are kinds of disinfection which have been utilized for millenia These methods have been supplemented by high temperature heating ultraviolet light and a variety of other forms of radiation including gamma rays and high energy electrons Chemical inactivation falls into two general categories agents with rather nonspecific toxicity such as Hg^{++} formaldehyde or the phenols and agents with highly selective interference of biochemical processes essential for growth such as the sulfonamides and the newer antibiotic agents Quite commonly the second group is considered bacteriostatic and the first bactericidal but this distinction is artificial since most of the nonspecific ger

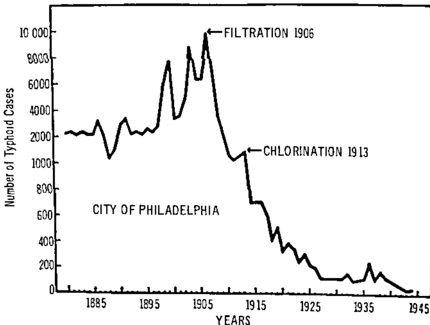


FIG 1 Reduction of typhoid fever in Philadelphia following treatment of the water supply (data supplied by Dr Angelo M Perri) From Smillie W G and Kilbourne E D 1963 Preventive Medicine and Public Health ed 3 p 134 New York Macmillan

and reinforcement of man s resistance to the agent

Neutralization of Reservoir

Destruction of Reservoir It is quite possible in some cases to eradicate an infectious disease by destruction of all infected animal hosts This method has been widely applied in the control of animal diseases such as tuberculosis of cattle and foot and mouth disease of cloven hoofed animals Plague may be eliminated at least in urban areas by a virtually complete eradication of the rat reservoir host Similarly rabies has been controlled in certain relatively isolated areas by the destruction of all identifiably rabid animals and the imposition of an effective quarantine system Total destruction of all infected animals may not be necessary A number of observations have suggested that when the proportion of infected animals in a population is reduced below a critical level the efficiency of transmission falls so low that the agent will not persist in the reservoir

Therapeutic Cure of Reservoir With the advent of chemotherapy and antibiotic therapy in the past 25 years the eradication of many diseases in which man himself is the reservoir has become both possible and feasible Thus yaws apparently has been eliminated from many previously highly endemic areas of the world by massive therapy with penicillin The limitations of this general approach for many diseases are great because frequently the individuals who are reservoirs for the agent are clinically healthy and are identifiable only through detailed and expensive laboratory tests The only alternative to selective identification of the reservoir individuals is treatment of the entire population and this is frequently neither feasible nor acceptable

Occasionally certain selected procedures (e g cholecystectomy in a typhoid carrier or tonsillectomy in a diphtheria carrier) may be of value in elimination of the reservoir (carrier) state in man Where such treatments are of value their beneficial effects are based on selective localization of the infecting agent in specific organs or tissues Usually they are employed only after other methods have failed to neutralize the reservoir

Isolation of Reservoir The isolation and the removal of obviously infected lepers from society may well be the oldest effective disease control program Quarantine has been employed for centuries in many logical situations and also in other circumstances which in light of present knowledge were totally illogical The need for such measures has been greatly reduced for many diseases which are susceptible to medications which render the individual noninfectious Further as with treatment programs aimed at curing the reservoir isolation and quarantine require the identification of the infected individual without symptoms as well as the individual with clinical disease Because such identification is difficult if not impossible in the face of an epidemic (e g of cholera), quarantine measures frequently require blanket application to a large number of uninfected individuals to control the relatively small number who are infected In spite of these limitations quarantine is still employed as an adjunct to other methods of control for example a person may be held in quarantine in areas endemic for smallpox cholera plague or yellow fever until immunization against the disease has been completed successfully Usually such drastic measures are limited to the major quarantinable diseases but they may be applied occasionally in less justifiable circumstances

Interrupting the Chain of Transmission

Mechanical Methods Under this heading fall a number of methods which provide limited benefits but are nevertheless useful in controlling the incidence of disease

Dilution Simple dilution of the vehicle of transmission may so reduce the number of organisms transmitted from the reservoir to the individual that the probability of infection becomes very slight In essence this is what is accomplished by increasing the distance between beds in military barracks bassinets in nurseries or seats in classrooms Similarly natural dilution of infected feces in bodies of water contributes in a major degree to reduction of the spread of waterborne agents Depending on the circumstances and the agents involved dilution may exert its effect by decreasing the average infective dose or by increasing the probability of kill

STERILIZATION AND DISINFECTION

INTRODUCTION

Factors deleterious to microorganisms range diversely from heat and cold to a variety of chemicals and radiations It is most unlikely that all of these factors operate through one common mechanism and yet there is a rather surprising uniformity in the effects which are produced

Two general levels of reactivity on the part of the microorganism can be identified The first is a response of suspended animation referred to as bacteriostasis in which most if not all of the metabolic activities of the cell come to a halt The organism respires minimally it does not grow it cannot reproduce and for all intents and purposes it is incapable of initiating or continuing a disease process However if the agent causing the bacteriostasis is removed or neutralized by the appropriate antagonist the organism rapidly regains all normal activity including pathogenicity In brief bacteriostasis is a reversible process within limits apparently caused by a selective chemical lesion which renders the cell incapable of normal activity There is usually little or no latent period between contact of the cell with the bacteriostatic agent and the response However if the condition of bacteriostasis is prolonged unduly it shades imperceptibly into the second microbial response to harmful factors

This second response is irreversible and results in the death of the cell In distinction to bacteriostasis the cell s response is not instantaneous With a majority of lethal agents it is dependent on both time and dose in a predictable manner Under suitable experimental conditions this response takes the form of a linear relationship between time and the logarithm of the number of cells surviving as follows

$$-\log_e n = kt \qquad (1)$$

n = number of cells surviving at time t and k = constant

The derivative of (1) yields the following expressions with transposition

$$-dn/n = kdt \qquad (2)$$

$$-dn/dt = kn \qquad (3)$$

That is the cells rate of dying is always proportional to the number of cells present

In the case of multicellular organisms the relationship demonstrated for microorganisms does not hold true Instead the results obtained for example with mosquito larvae and a toxic substance such as heptylic acid yield an obviously concave curve when plotted in the same fashion that yields a straight line for microorganisms

The reasons for the difference between the responses of unicellular and multicellular organisms have been the subject of extended scientific examination and discussion well summarized in the review by Rahn (1945) The evidence suggests that the difference exists because of the simplicity of the unicellular system in which the whole organism reflects the inactivation probably by a single hit of one of the genes responsible for the processes involved in continued multiplication of the organism With multicellular organisms particularly the vetebrates the life of the individual is not dependent on the viability of the genetic system responsible for propagation but rather on the viability of an interlocking complex of many cells responsible in toto for continuation of the metabolic processes of the organism as a whole

The results obtained in the process of disinfection are also influenced by temperature In common with other chemical reactions the Q_{10} values for most disinfectants approach 2 i e the rate of the reaction is doubled for a 10 C increase in temperature In practice this principle may be applied to make feasible the use of lower concentrations of disinfectant at elevated temperatures for disinfection in a fixed time interval or reduction of the time required for action at the same concentration (Rahn, 1945)

This information is of more than merely theoretic interest because it defines the results which may be expected when we attempt to kill microorganisms These results apply equally well to dressings and intravenous fluids sterilized in an autoclave to catgut sutures sterilized by radiation or to diphtheria bacilli killed by phenol For these reasons an understanding of the death/time relationship is important to all who practice medicine

micides can be bacteriostatic in sufficiently high dilutions Chemical methods for interrupting the transmission of disease agents have many applications Chlorination of water supplies the addition of mercurial or quaternary ammonium preservatives to biologic products and the application of organic iodine complexes to cuts and abrasions of the skin are a few examples

Biologic Methods Interruption of transmission by environmental measures aimed at living vectors of disease is a highly specialized topic which can be touched on only briefly here Control of malaria and yellow fever are probably the most dramatic examples of this type of control measure Vector control may be aimed at reducing the breeding potential of the mosquito or other arthropod involved in the transmission of disease or it may be aimed at interrupting the movement of the adult vector as it moves from an infected host to a susceptible person Using malaria as an example the first approach may include water drainage manipulation of water level or application of insecticides to the water surface The second—and more effective measure in the case of malaria at least—is exemplified by the use of residual long acting insecticides in human dwellings

Protection of the Susceptible Person

A number of the procedures mentioned above may be used to protect the susceptible individual or group as well as to prevent the spread of infection from the infected person or group A cardiac patient may be denied visitors during an influenza epidemic or may wear a mask instead of requesting the visitors to do so However far more effective measures for protecting the susceptible individual are those which directly alter his relative capacity to deal with a specific infection if he is exposed to it There are several such protective measures

Chemoprophylaxis It is probable that chemicals of one sort or another have been ingested or applied locally since early times in an effort to prevent the acquisition of infection Probably the best established chemoprophylactic agent is quinine from cinchona bark used by South American Indians for the prevention of malarial fever long before the nature of such fevers was understood However, the major advance of chemoprophylactic measures began with the introduction of the sulfonamide drugs followed by penicillin and the broad spectrum antibiotics Their application to the control of various infections will be discussed in greater detail below

Immunization Since time immemorial man has recognized that with a number of diseases second attacks occurred rarely if ever and has concluded rationally that recovery from the disease produced what we now refer to as specific immunity Efforts to simulate this immunity by artificial technics which are designed to produce resistance with less risk than the actual disease date back to the use of smallpox crusts for immunization of infants (mentioned above) The list of the diseases of man for which artificial immunizing agents are now available is impressively long and is growing steadily It is possible that more lives have been saved by immunization against smallpox, for example than by any other single measure for the control of infectious disease Although some generalizations are applicable with regard to immunization many others are extremely hazardous or inaccurate The guiding principles and the variations in the patterns observed are discussed in detail below

Enhancement of "Natural Resistance" The known factors pertaining to natural resistance to infectious diseases have been discussed in several of the early chapters in this volume Although many artificial ways of *impairing* these mechanisms have been employed (e g by depressing the number and the activity of phagocytes) relatively few mechanisms for *enhancing* them have been discovered A great variety of nonspecific stimulating substances have been employed without any outstanding success and with little understanding of the underlying principles

Microbial Interference As noted in the discussion of antibiotics in Chapter 40 the presence of certain microbial organisms may interfere with the establishment of others The application of this principle to the prevention of infections is still in its infancy but merits brief notice

more effective in sterilization than dry heat this observation parallels the observed fact that native proteins are denatured (coagulated) at higher temperatures as their water content decreases

In practice the autoclave is basically a sturdy metal container which can be sealed tightly and filled with steam at elevated pressure The common pressure cooker is the simplest autoclave and is capable of providing sterile materials in emergency situations The common autoclave found in medical installations is a horizontally mounted cylinder with a door at one or both ends and may vary in size from one just big enough to sterilize a small tray of instruments to one big enough to handle bed mattresses Regardless of size each installation should include the following features (1) a source of saturated steam under pressure either integral to the unit or in the form of a steam line (2) a pressure gauge at the steam inlet at the top of the chamber (3) a safety valve to prevent explosion due to overpressure (4) an air vent and a shutoff valve connected at the lowest part of the chamber and (5) most important, a reliable thermometer connected in the exit line Desirable but not essential features would include (1) a jacket external to the chamber to provide additional heat and (2) a means of connecting the chamber to a slight vacuum including adequate microbial filters for readmitting sterile air to the chamber

The cardinal fact in effective use of the autoclave is that live saturated steam *must* come into intimate contact with every part of the contents of the autoclave Thus items to be sterilized must be wrapped in coverings that are freely permeable to steam and yet of fine enough weave to prevent subsequent contamination of the sterilized article by dust or vermin Double thickness muslin has been the covering of choice for many years but is being replaced by high strength porous paper in many locations Regardless of the manner of covering the items placed in an autoclave must be packed loosely with sufficient space between them to allow free penetration by steam

Because steam is lighter than air careful attention must be paid to the positioning of any container flask, tin or drum in which air may collect and prevent contact of the steam with the full surface of the container and its contents Such containers *must* be placed in such a position that all air may drain *downward* from their mouths or they must have sufficient (about 5 ml /L volume) water within them to expel residual air by vaporization during the heating cycle Air is expelled from an autoclave by *downward* displacement if air is retained within the autoclave sterility of the load cannot be assured Flasks and tubes containing media or solutions in appreciable volume require no additional precautions other than providing sufficient time to permit heating the contents to the required temperature for the required time

As steam fills the autoclave the material in it is heated by condensation of water vapor with the release of 540 cal / Gm of moisture condensed The temperature of the load if properly packed rises to 100 C in a relatively short time As soon as the exit line thermometer reads 100 C all air will have been displaced from the chamber and the exit valve may be closed (This function is frequently performed automatically by a thermostatic valve Since this valve may fail under use a routine check on the temperature at the exit line at the time of closure is advisable)

Practically all autoclaves are equipped with a steam ejection pump to provide a slight vacuum to assist in drying the load The negative pressure achieved is minimal—on the order of 20 inches of H O or 40 mm Hg *below* atmospheric Nevertheless even this slight vacuum is sufficient to increase greatly the vaporization of moisture from the hot load This common provision for drying materials after autoclaving should not be confused with the high vacuum assisted autoclave referred to later

Although the length of time required to sterilize a load at a particular temperature and pressure will depend on both the volume and the nature of the load it is possible to specify the following equivalents as the minimal time/temperature relationships to be maintained throughout a load to assure sterility (Medical Research Council 1959)

A number of indicator systems are available which will indicate that a given temper

The most important and to many the most unexpected implication of the death/time relationship is revealed by equation (2) above In plain words, that equation states that the *proportion* of organisms dying in equivalent time periods is a constant If a number is successively reduced by half in a series of operations simple mathematics tells us that it is impossible however long the operations are continued to reach zero although for all practical purposes that is the limit which is *approached* The situation is precisely analogous in sterilization and the implication of this fact for medical and biologic problems has never been better stated than by Powell In regard to the probability function implied in the logarithmic death/time relationship of sterilization he wrote

> This is characteristic of all methods of sterilisation—heat ethylene oxide chemical disinfection Thus it will be seen that as the length of treatment increases the probability of finding any viable microorganism decreases without theoretically reaching zero i e absolute sterility This is not generally realised unfortunately particularly in medicine and pharmacy but it is well recognised in the food industry This does not mean that we are questioning whether an item is sterile or not but rather how many items can be sterilised under a particular set of conditions before one of them will be found to be non sterile *

With this introduction the next sections will be devoted to detailed consideration of the processes of sterilization and disinfection used in medical and biologic work The comments in regard to the death/time relationship apply throughout except for the mechanical methods of which filtration is the best example

STERILIZATION

Sterilization is the elimination of *all* viable microorganisms by heat radiation chemical compounds or mechanical methods It is an absolute term unqualified by whether the organisms are pathogens or commensals

The term sterilization (in the microbiologic sense) came into being with the demonstration of the role of microorganisms as the causative agent of putrefaction —a result of the studies of Jablot Spallanzani Pasteur and others in the spirited controversy in regard to spontaneous generation Only with demonstration and acceptance of the dictum that like comes from like was it possible to define the processes of sterilization and disinfection This event was followed by the rapid growth of the field of pathogenic microbiology leading by the last part of the 19th century, to technics media and taxonomic classifications suitable for critical studies of the process of sterilization Since that time knowledge of the process has evolved until today the term sterilization includes the destruction of all forms of microorganisms whether they be vegetative cells spores or virus particles

Sterilization is defined as successful if appropriate tests cannot demonstrate *multiplication* of the organisms originally present Thus the criterion of viability of a microorganism the ability to multiply is much simpler than the corresponding criterion for the human organism for example Its simplicity is deceptive Koch s original studies on the inactivation of anthrax spores are a case in point His estimates of the killing power of $HgCl_2$ for this organism were subsequently found to be too high because spores which had been sterilized could multiply if the organisms were first treated with H_2S to remove the toxic Hg^{++} ions in the form of the relatively insoluble HgS Another example of an overestimation of the sterilizing power of a chemical agent may be found in the recent experience with killed poliomyelitis vaccine (Nathanson and Langmuir 1963 Wilson 1963) The reader is referred to the cited references for a discussion of the factors involved

Methods of Sterilization

Moist Heat HIGH PRESSURE STEAM STERILIZATION The use of high pressure steam in an autoclave is the most satisfactory method of sterilization in common use today Death due to moist heat is attributed to heat denaturation of protein constituents of the microbial cell but nothing is known of the precise injury responsible for death It is generally recognized that moist heat is far

* Powell D B Application of radiation sterilisation to surgical materials *in* Recent developments in the sterilisation of surgical materials Report of a symposium pp 9 10 London The Pharmaceutical Press 1961

more appropriately as chemical disinfectants Second the mechanical equipment required to utilize the chemical sterilants is similar to that found in the vacuum assisted autoclave Indeed many commercial installations of sterilizing equipment are combination gas steam sterilizers

Ethylene Oxide The simplest cyclic ether ethylene oxide (C_2H_4O) is a colorless gas with a pleasant ethereal odor It liquefies at 10 8 C and solidifies at —113 3 C It has a flammability similar to that of diethyl ether and a similar disturbing property of forming highly explosive mixtures with air over a wide range of dilutions Within the past decade several firms have made available chamber sterilizers designed for use with ethylene oxide which have practically eliminated the explosion hazard associated with this form of sterilization The safety of the equipment depends in part on mechanical design and in part on the use of non flammable mixtures Two diluents have been used in proportions of 9 parts of an inert gas to 1 part of $C H_4O$ Carboxide® is the trade name of the mixture with carbon dioxide Cryoxide® is that of the mixture with freons At present ethylene oxide sterilization is attracting strong interest in the medical field particularly for the class of materials that cannot withstand the heat of autoclaving (Mayr 1961 Perkins and Lloyd 1961 Phillips 1961) As greater experience is gained with this method it will undoubtedly find greater application in medicine although it is never likely to become as widely used as the autoclave

The sterilizing action of ethylene oxide is attributed to its high chemical reactivity in alkylating sulfhydryl amino carboxyl and hydroxyl groups in the protein molecule (Phillips 1961) Recent studies have demonstrated that rapid sterilization with this agent requires a relative humidity of 30 per cent or greater suggesting that the water content of the microorganism is important in the action of the sterilant Recommended exposure times at 50 per cent relative humidity and 54° C are 5 hours at 450 mg /L or 3 hours at 900 mg /L (Perkins and Lloyd 1961)

In practice ethylene oxide sterilization is similar to the use of the vacuum assisted autoclave After loading and sealing the chamber a vacuum is drawn and carefully measured volumes of water and diluted ethylene oxide are admitted to achieve the desired concentrations of both The chamber remains at a slight negative pressure for the duration of the sterilization cycle and heat is applied to bring the load to a temperature of 54 C Penetration of the gas is rapid even through sealed plastic containers and packages At the end of the sterilization cycle the chamber is again evacuated to remove residual gas Because ethylene oxide dissolves to an appreciable extent in rubber and plastics (as a gas in solid solution) a further waiting period is required before use of such items in order to permit diffusion of gas from the material (Perkins and Lloyd 1961) In all a full cycle from loading to release for use of such items as rubber gloves requires about 24 hours so that it is not a particularly rapid process On the other hand for items made of plastic rubber or other thermolabile materials ethylene oxide sterilization is very attractive because it results in minimal deterioration The process leaves little to be desired in terms of efficiency provided that the requirement for 50 per cent relative humidity is met Indeed many medical items on the market today have been sterilized by this means

It is of some interest that the use of ethylene oxide as a disinfectant and sterilant is not new except in the medical field It has been employed in the tobacco and the spice industries for at least 30 years (Mayr 1961) The gas is not without untoward effects on certain chemicals as might be expected from its chemical reactivity It has been found that animal feeds that are sterilized with ethylene oxide suffer marked destruction of both vitamins and amino acids (Windmueller *et al* 1956 Windmueller *et al* 1959) On the basis of these results caution should be exercised in the use of ethylene oxide in sterilization of bacteriologic media, even though this application has been described (Wilson and Bruno 1950 Judge and Pelczar 1955)

Formaldehyde The simplest aldehyde formaldehyde (CH_2O) is a pungent chemical that is familiar to most individuals in medicine because of its wide use in preservation of anatomic materials It has had a long

TABLE 1 TIME/TEMPERATURE RELATIONSHIPS FOR STERILIZATION WITH SATURATED STEAM

TIME MIN	TEMPERATURE C	PRESSURE LB /IN 2
15	121	15
10	126	20
3	134	30

ature has been reached during sterilization (Perkins 1957) One in very common use is a recording thermometer in the exit line but unfortunately this device provides no data on the temperature of the load At least two systems will show that the load has been exposed to a minimum temperature for a minimum time period However all of these methods of measurement are subject to criticism Perhaps the most satisfactory control system for sterilization is the use of a test strip inoculated with a sporulating organism with known thermal resistance characteristics Even this technic has the disadvantage that it provides no *immediate* answer to the question of whether the load has been subjected to adequate heat for an adequate time to sterilize the test organism Nevertheless it does provide valuable information that is not available from the other methods and it is frequently employed (in addition to simpler less definitive methods) as a weekly or monthly check on the conditions of sterilization at many institutions

There has been considerable interest in shortening the time required to complete the sterilization cycle Greatest attention has been directed to the initial air displacement phase since frequently as much time may be spent in removing air from the chamber as in the high temperature phase of the cycle This has led to the development of the vacuum assisted autoclave *in which the* chamber is exhausted to an *absolute* pressure of 20 mm Hg or less before steam is admitted (Medical Research Council 1960 Bowie 1961 Shotton 1961) Such technics pose several problems of mechanical design of chamber and door to withstand both positive and negative pressures and also of adequate microbial filtration of the air which reestablishes atmospheric *pressure* within the chamber Several units are now commercially available and are of value in such applications as emergency sterilization of surgical instruments

With any installation extreme care must be exercised in making pressure changes when fluids are being autoclaved Unless these changes are sufficiently gradual plugs and stoppers may be blown from containers or glass bottles may be violently shattered by explosive boiling of superheated liquids Despite these minor problems—which need only to be understood by the operator—sterilization by steam under pressure remains the most frequently used method of sterilization in medical practice today

INSPISSATION AND TYNDALLIZATION Both of these technics have been used for the preparation of certain bacteriologic media and find limited use even today Inspissation i e the coagulation of serum or proteinaceous media by short exposure to temperatures of from 85 to 100 C is still utilized in many laboratories for the preparation of Loeffler s blood serum agar for cultivation of diphtheria organisms It is usually followed by autoclaving or tyndallization Tyndallization also called fractional sterilization may be used as an emergency substitute for more efficient methods or with those few media which cannot withstand the temperature of autoclaving As originally described it consisted of heating at 80° C for 30 minutes on each of 3 successive days The technic now has a number of variations in both temperature and time e g 100° C in flowing steam for 60 minutes on 3 days Regardless of the method employed the rationale is the same An exposure at 80° C for 30 minutes is adequate to kill all vegetative forms present Any spores surviving the initial heating should germinate by the second day and be killed in the second heating The third heating provides *a safety factor for a slightly marginal* process

GASEOUS STERILIZATION Although this method logically might be included under the heading of chemical agents it is considered at this point for two reasons First there are only three chemicals—ethylene oxide formaldehyde and β propiolactone—worthy of consideration as chemical sterilants The remainder of the chemical agents are classed

ature required for sterilization (as in the pro cedure recommended for the autoclave) Provided that such a control test is included it is possible to define the time/temperature relationship for any object to be sterilized by dry heat The usual limits recommended for glass and metal items are 2 hours at 160° C or 3 hours at 140 C The time of exposure starts with the time the most central part of the load reaches the indicated temperature so that these limits do not include the time required to bring the load to sterilizing temperature

INFRARED A second type of dry heat sterilization which has achieved acceptance in the large scale processing of syringes in Britain is the use of infrared heating lamps in an enclosed conveyer belt process (Hopkins 1961)

FLAMING AND COMBUSTION The flaming of the microbiologist s loop to incinerate viable organisms is familiar to everyone who has worked in the laboratory and represents an extreme example of heat sterilization by combustion Incineration is also sometimes employed in the air exhaust systems used with enclosed hoods for handling highly infectious agents in the laboratory On the other hand the routine flaming of the mouths of culture tubes and flasks by the microbiologist would appear to be of very little value in sterilizing these surfaces because of the short period of exposure It is possible that the practice has some benefit but, if so it is more likely to be that of burning off cotton fibers or dust that otherwise might fall onto the surface of the culture medium

Radiation GENERAL Three types of radiation have assumed importance in the sterilization of biologic materials ultraviolet light (UV) gamma (γ) and x ray radiation and high energy electrons (β particles) Although these differ from each other in both nature and energy the processes by which they damage the microbial cell are similar The UV the γ and the x ray radiations are electromagnetic in nature and all follow the laws of quantum mechanics When they are absorbed by biologic material ene gy is released in packets or quanta the energy content of which is inversely proportional to the wavelength of the radiation The same quantal release of energy occurs with the absorption of visible light although the energy package in this case is not sufficiently large to cause damage it may be used in plants (for example) for photosynthesis In the transition between violet visible light and the UV a threshold is crossed and the energy of the quantum absorbed becomes large enough to induce ionization within the cell The changes that result from such ionization depend on the energy released as well as the morphologic site of its release With marginally effective quanta of absorbed UV chemical bonds may be ruptured and new bonds formed or the energy may be dissipated totally as heat without chemical changes As the wavelength of the incident radiation decreases to that of γ or x ray radiation the energy released per quantum becomes hundreds to thousands of times greater than that per quantum of UV Accordingly there are very few such high energy quanta which are inactive in the sense that they produce only thermal effects Chemical changes become more drastic and far reaching The energy of the quantum is dissipated among many molecules along its path and may produce drastic chemical changes in more than one cell Such changes may take the form of genetic changes and if the change is lethal may result in the death of the cell

High energy electrons (β rays or particles) are particles in nature and differ in this respect from UV and γ radiations Nevertheless such particles when accelerated to nearly the speed of light produce much the same effects in cells as do UV and γ radiations The electrons gradually release their energy through multiple collisions in the atomic matrix of the cell and produce chemical changes similar to those produced by γ radiation (Trump 1961)

GAMMA RADIATION Cobalt 60 sources have been the most commonly used gamma emitters in sterilization installations to date This technic requires radiation sources of 30 to 100 kilocuries* which are far larger than most cobalt 60 sources used for therapy and therefore require elaborate shielding and

* A curie is the unit of radioactivity defined as that quantity of radioactive material producing 3 7 $\times 10^{10}$ disintegrations/second 1 kilocurie = 10^3 curies.

and colorful history as a preservative and a disinfectant, but as a gaseous sterilant it runs a poor second to ethylene oxide

The sterilizing action of formaldehyde is attributed to its reactivity for the amino groups in protein molecules Precise data on exposure times required for sterilization are difficult to obtain and a few remarks on the chemistry of the material may provide an explanation Formaldehyde as a chemical is characterized by a propensity for polymerization and a high affinity for water Indeed concentrations of the free gas in air usually do not exceed 1 75 to 2 0 mg /L Once this concentration is exceeded the gas condenses as a poorly defined polymer paraformaldehyde Under certain conditions the gas may also form a ring condensation product, trioxane Both paraformaldehyde and other condensation products break down on heating to formaldehyde Formaldehyde is also available commercially as formalin, a solution of 37 per cent formaldehyde and 5 to 10 per cent methanol in water The gas in solution exists to a considerable extent as a chemical hydrate which may be broken down by heating With this background it is understandable that both temperature and relative humidity have been found to be major influences on the sterilizing action of formaldehyde It is also understandable that formaldehyde slowly vaporizing from condensed paraformaldehyde may be released for appreciable periods of time by articles exposed to the gas

Because of the characteristics mentioned above formaldehyde has found little popularity as a sterilizing agent It has been utilized in the disinfection (and perhaps sterilization) of both sick rooms and laboratory areas Under such use it has been recommended that 1 ml of formalin be vaporized (either by heating or by spraying from a fine nozzle) for each cubic foot of space involved An exposure time of 10 hours has been recommended at a temperature of 70° F and 50 per cent relative humidity An airing period of several days usually is required to rid the room of the odor of the gas (Phillips 1957) Formaldehyde also has been used in the sterilization of certain types of medical and surgical equipment that would be harmed by heat

Beta propiolactone (C_3H_4O), a condensation product of ketene and formaldehyde has been employed in aqueous solution to sterilize biologic materials and in vapor phase to decontaminate items of medical equipment (Hoffman and Warshowsky 1958, Allen and Murphy, 1960)

Dry Heat HOT AIR OVEN Second to the autoclave the hot air oven is one of the important sterilization devices in use today It has had a long history dating back to the last century when it was first used in microbiology However even at that time Koch recognized that dry heat was much less effective than moist heat in sterilization His observations were paralleled by the observations of the biochemists that enzymes were far more resistant to heat in the dry state than they were in solution The exact mechanism by which dry heat inactivates microorganisms is unknown but it has been suggested that it is mediated through an oxidation process (Perkins 1957)

For these reasons a temperature of 160° C for a period of 1 to 2 hours is necessary in a hot air oven to achieve the same conditions of sterility that may be obtained at 121° C in 15 to 30 minutes in the autoclave Not only is a longer time required to achieve the same result but since all heating is by convection and radiation unassisted by the heat of condensation of water vapor it will usually require a longer time for a load of equipment to reach sterilizing temperature in the oven than in the autoclave In spite of these limitations and the further problem that both cotton plugs and cloth are somewhat weakened by exposure to 160 C the dry air oven is the preferable method of sterilization of many metal or glass items heat stable powders and other materials which would either react or be damaged by exposure to water vapor or condensate

The method of loading a hot air oven is fully as critical as that for the autoclave Because of the less efficient heat transfer by air even when under forced convection by fan adequate space for air circulation must be provided between packages on a single shelf and between shelves in the oven If it is necessary to sterilize large porous items by dry heat a spore control should be included in the initial tests to determine the time and the temper

fluids is an old technic to which new materials have been applied within the past decade The first filters were made of porcelain by Pasteur and Chamberland in the form of a cylindrical candle Subsequently German manufacturers introduced a similar filter made of diatomaceous earth Both types were fragile and difficult to clean following use but nevertheless permitted the separation of microorganisms from their suspending fluid They played an important role in the early work with bacteriophage and until the early part of the 20th century were the only filters available Then the asbestos pad (Seitz) filter the sintered glass filter and the collodion and nitrocellulose graded porosity ultrafilters (Gradacol and Millipore®) were introduced Each of these types of filters can give excellent results and each has its own advantages and drawbacks

The physical process of filtration cannot be likened simply to the use of a sieve in separating various sizes of sands and gravels It was early demonstrated that most bacterial filters carry a negative charge and since bacteria themselves carry a negative charge at physiologic pH s forces of electrical repulsion are present All filters also adsorb solutes particularly proteins from the material that is filtered and this tends to decrease the effective pore size All in all at least three factors—mechanical sieving electrical repulsion and solute absorption—are at work with most filters so that the dimensions of the organism retained by the filter are smaller than the pores of the filters Filters must be sterilized before each use and once filtration is initiated it must be completed within a reasonable time Otherwise organisms retained by the filter will multiply and in time grow through the filter from the contaminated to the sterile face Simple tests are available using either a test microorganism or physical means for checking the integrity of a bacterial filter For details the reader is referred to the summary by Sykes (1958)

Ultrasonic Treatment This method of treatment is mentioned in passing not because it has practical value in sterilization but because it is both a research tool and a practical method of cleansing items of surgical and laboratory equipment Frequencies of 10 kilocycles to several megacycles have been employed In every case ultrasonic energy has been applied to a fluid medium in the form of longitudinal mechanical vibrations At high energy levels such vibrations both heat the fluid and produce cavitations — minute cavities in the fluid which expand and collapse in response to the pressure changes of the ultrasonic wave propagated through the fluid These cavitations are exceedingly effective in cleaning the surfaces of objects immersed in the fluid Furthermore bacteria particularly the rod shaped organisms are susceptible to rupture by the stresses produced by the cavitations The spherical bacteria are relatively resistant to rupture so the technic has not found application in sterilization However for certain organisms it is a convenient and practical method of releasing bacterial contents obtaining cell walls and rendering the majority of organisms non viable (Sykes 1958)

Freeze thawing This technic like ultrasonic exposure has little general application in sterilization but is of interest because it may be valuable for destruction of certain fragile organisms e g amoebae Disruption is caused by mechanical stresses resulting from the formation of ice crystals in the suspending medium Crystalline ice is subject to migratory recrystallization i e increase in size of large crystals at the expense of small ones at temperatures not far below 0 C This crystalline rearrangement also can produce mechanical damage to microorganisms It is for this reason that cultures of microorganisms are usually better preserved at temperatures of —70 C than at —20 C The topic of freezing drying and preservation of biologic materials has been summarized in a recent monograph (New York Academy of Sciences 1960) to which the reader is referred for greater detail

Disinfection and Antisepsis

Disinfection is the destruction of microorganisms on inanimate objects most commonly by chemical means Antisepsis is closely related but carries the connotation that the destruction is accomplished on a body surface Thus it is possible to consider a given agent as either a disinfectant or an antiseptic depending on the method of usage

safety precautions With radiation sterilization as with most other forms of sterilization, microbial spores and viruses require higher dosage levels than do vegetative cells Experience has accumulated to indicate that a dosage level of 2 0 to 2 5 megarads* is sufficient to reduce the surviving organisms by a factor of 10^7 which has usually been accepted as sufficient for medical applications Larger doses may be required with certain microorganisms or with certain materials

Side reactions at the 2 5 megarad dosage levels are minimal It is not possible to induce radioactivity with the 1 17 and 1 33 MeV† radiations emitted by cobalt 60 but slight deterioration has been noted in products made of rubber wool or plastic In practice gamma sterilization has been applied to dressings syringes catheters and other medical items It is also attracting attention as a means of preserving foods

HIGH ENERGY ELECTRONS Linear particle accelerators such as the Van de Graaff generator have been utilized in the commercial preparation of surgical sutures and other materials for a number of years Peak energies obtainable by the direct particle accelerators are limited to about 5 MeV in production installations but greater energies are obtainable through use of the indirect accelerators such as the microwave linear accelerator Peak energies above 10 MeV are likely to induce some radioactivity in the product sterilized and for this reason most work has been done with generators in the 1 to 5 MeV range In general a dosage level of 2 0 to 2 5 megarads is required with high energy electron radiation to achieve a 10^7 fold reduction in microorganisms and in this respect γ radiation and β particle sterilization are comparable They are also comparable in respect to side-effects in production of minor deterioration of rubber wool and plastic (Trump 1961)

ULTRAVIOLET RADIATION UV light has been studied for a number of years since the development of the carbon arc and the mercury vapor arc lamp It is far less energetic than either γ radiation or high energy electrons the UV wavelength of the mercury line at 2537 Angstrom units‡ corresponds to a quantum (or photon) energy of only 5 eV Accordingly, UV radiation has a very low capacity for ionization or excitation of molecules

Nevertheless even the UV radiation in sunlight has appreciable killing power readily demonstrated by exposure of an inoculated plate to direct sunlight For this reason the past two decades have produced much interest in the use of UV light to sterilize air in laboratories surgical operating rooms and classrooms Unfortunately, effective use of UV light as a sterilizing agent is limited by the dermal burns it produces in man and by its extremely limited penetrating power even into most substances which transmit visible light Although it is quite possible to kill many organisms by exposures to 5 to 10 milliwatts/cm² in time periods of 1 minute or less the maximum allowable level for human exposure has been set at 0 5 and 0 1 microwatts/cm² for 8- and 24 hour exposure periods (Shechmeister 1957) Whenever UV intensity levels have been brought within these limits in application to classrooms and hospital wards little practical value has been recognized in terms of reduction of illnesses or major reductions in bacterial counts In situations in which high intensity UV radiation can be employed—e g disinfection of a laboratory work area following use—much more satisfactory results have been obtained Similarly good results have been reported in the use of high intensity UV barrier' ir radiation in hospital situations where patients are restricted to the areas so protected (Wells 1955) UV like light travels in straight lines and for this reason cannot penetrate into cracks and hidden corners The current opinion is that its usefulness is limited and an excellent annotated summary has been prepared by Sykes (1958)

RADIO FREQUENCY AND INFRARED These forms of radiation have no intrinsic capability of sterilizing biologic materials other than through thermal effects which both produce (Sykes 1958)

Mechanical Methods FILTRATION The use of mechanical filtration for sterilizing

* A megarad is 10^6 rads A rad is defined as a unit of radiation equivalent to the deposition of 100 ergs of energy/Gm of absorbing material

† MeV = 10^6 eV 1 eV = work done in acceleration of one electron by a 1 volt field

‡ Angstrom unit = 1/10 000 micron

ity of the milk To meet this objection a high temperature short time pasteurization by heating to 71 7° C (161° F) for 15 seconds had previously been developed and this flash pasteurization treatment will destroy the agent of Q fever Any of these methods will also inactivate a phosphatase present in untreated milk so that the assay for this enzyme is frequently employed as a confirmatory test for pasteurization

Chemicals PHENOLS SOAPS ALCOHOLS AND RELATED COMPOUNDS *Phenol* is one of the oldest disinfectants intentionally used in medical practice having first been used by Lister in surgery in 1865 In comparison with other agents available today it is relatively ineffective as well as moderately toxic to living tissue Because of its position in the field of disinfectants it has been adopted as the standard against which other agents are tested The activity of phenolic compounds is increased by halogenation or the addition of alkyl side chains The alkyl side chains produce surface active agents in which the alkyl chain becomes oriented to the lipid phase and the hydroxy group to the aqueous phase of an interface In general this class of compounds is effective against vegetative forms of bacteria including the tubercle bacillus and the fungi Activity against spores and viruses is minimal The mode of action of the phenolic detergents is mediated either through the ability of the agents to denature proteins or through their ability to increase the permeability of cell walls (Gale and Taylor 1947)

In practice the phenols are frequently used as mixtures of a phenol (such as tricresol) in soap Such mixtures are both excellent cleansing agents and moderately good disinfectants and this has undoubtedly contributed to their popularity However too much soap can actually eliminate the germicidal action of the phenol by selective adsorption of the disinfectant on the soap micelle One of the substituted phenols hexachlorophene or G 11 retains its full activity in soaps and is widely used in proprietary surgical soaps This compound is active against the majority of the gram positive skin contaminants but it is relatively inactive against gram negative organisms and for this reason should be limited in usage to skin cleansing and disinfection (Sykes 1958)

Soaps themselves have only moderate activity as bactericidal and bacteriostatic agents against microorganisms in addition to their purely physical action in cleansing In general their activity varies with the fatty acid component (Sykes 1958)

Anionic detergents such as sodium lauryl sulfate have minimal antimicrobial action except in high concentrations and this is primarily against gram positive organisms

Alcohols have had a long history in disinfection but relatively few have achieved an important place The two most common alcohols in everyday use are ethyl alcohol and isopropyl alcohol Both are effective against vegetative cells slowly effective against viruses and almost totally ineffective against spores Ethyl alcohol is most effective as a general disinfectant in concentrations of 50 to 70 per cent It is being replaced by isopropyl alcohol in many applications since the latter compound is not subject to legal restrictions The alcohols apparently act through protein denaturation

Ethylene and propylene glycols have received considerable attention as air disinfectants They are appreciably active as thermally generated aerosols against many viruses and bacteria under experimental conditions when the relative humidity is held between 40 and 65 per cent Ethylene glycol is effective in about 1 part per million (p p m) in air viruses tested and shown to be susceptible to its action have included influenza meningopneumonitis psittacosis mumps and Newcastle disease (Sykes 1958)

DYES Two major classes of dyes exert bacteriostatic effects particularly against the gram positive organisms Some of the members of the triphenylmethane series have such selectivity in their activity that they have been incorporated into selective media for growth of the gram negative organisms i e brilliant green and crystal violet This group of dyes produces bacteriostasis by interfering with the oxidation mechanisms of the cell but the exact nature of the inhibition is still speculative (Fischer and Munoz, 1947)

The other major group of bacteriostatic dyes is the acridines Like the triphenylmethane series the acridines interfere with the

In discussion of the disinfectants and antiseptics two suffixes are commonly employed The suffix cidal indicates a lethal effect e g, bactericidal virucidal or fungicidal whereas static indicates inhibition of multiplication without lethality e g bacteriostatic virustatic or fungistatic

Disinfection of articles of commerce and particularly articles of mail dates back about 500 years The interested reader is referred to the monograph of Meyer (1962) for a detailed review of postal disinfection The intentional medical use of antiseptics is more recent in origin Semmelweis advocated chloride of lime for preventing the spread of puerperal sepsis and Lister chose phenol for his early efforts in antiseptic surgery

Dynamics of Disinfection

The time action relationship discussed in the introduction applies to disinfection and antisepsis as well as to sterilization Where chemical disinfection is concerned the nature of the suspending medium is critical in appraising the activity of the agent This should be expected because most disinfectants react with proteins in general and other nitrogenous materials in the test medium compete with the microorganisms for the disinfectant Testing the activity of disinfectants is a complex procedure and for this reason no attempt will be made to discuss it in detail In principle a new disinfectant is compared with a standard (usually phenol) to determine antimicrobial activity in a given time interval The ratio of the dilution of the new disinfectant to the dilution of the phenol giving comparable activity is termed the *phenol coefficient* Since many disinfectants are bactericidal in low dilutions and bacteriostatic in high dilutions erroneous conclusions may be reached unless the bacteriostatic effect is eliminated either by extreme dilution or by a specific antagonist (such as H_2S for $HgCl_2$) The topic of disinfectant testing is thoroughly dealt with in two excellent texts (Reddish 1957, Sykes 1958)

Methods of Disinfection

Moist Heat BOILING Moist heat in its simplest form i e boiling, has been widely used for disinfection It destroys vegetative forms of most microorganisms in a brief exposure, and even spores of most human pathogens are destroyed within a few minutes at 100° C Until the past decade this method of disinfection has been widely used in physicians' offices for syringes needles and small instruments With the demonstration of the transmission of the virus of infectious hepatitis in serum-contaminated needles this practice has been largely discarded for more efficient methods, e g autoclaving However, there may be emergency situations where boiling is the only method available to disinfect equipment and, if so it should be borne in mind that less than 30 minutes at 100 C may be insufficient to inactivate the infectious hepatitis virus (Eichenwald and Mosley 1959) Boiling does not sterilize in the correct sense of the word since many nonpathogenic organisms in spore form may survive exposure to 100° C for 30 minutes Because these organisms are incapable of producing disease in man they are of no concern if the material so treated is to be used for injections or minor surgery From the microbiologist s viewpoint such organisms are capable of growth in culture media and a single boiling is inadequate for his purposes without the added refinement of tyndallization (described under Sterilization)

PASTEURIZATION This procedure employed primarily for treating milk and other fluids is another example of selective elimination of human pathogens without destroying a large number of microorganisms whose presence may be accepted as noninjurious As originally developed by Pasteur pasteurization was designed to eliminate undesirable bacteria which oxidized to acid the alcohol produced in yeast fermentation of beers and wines That application of the technic was not generally accepted at first, allegedly on the grounds that it spoiled the true flavor of the beverage The process subsequently was applied widely in the milk industry to eliminate the agents of tuberculosis and brucellosis from contaminated milk Recent studies have indicated that conventional pasteurization at 61 7° C (143° F) for 30 minutes will not kill the organisms of Q fever if they are present whereas a temperature of 62 8° C (145° F) will do so (Enright 1957) Unfortunately the higher temperature has an adverse effect on creaming abil

for disinfection The conventional chemical test for determining chlorine levels in water supplies and swimming pools the orthotolidine test gives results in terms of total residual chlorine which if the water contains appreciable ammonia may erroneously indicate a safe level of chlorination when in fact *the water is unsafe There is as yet no simple* test for free chlorine but the need for further research in this area is widely recognized

The exact means by which HOCl exerts its bactericidal action is unknown but it is apparently by oxidation of the cell membrane There is no doubt that the material is bactericidal rather than bacteriostatic The disinfecting action of chlorine containing compounds is highly dependent on pH of the water and temperature Further such variation of activity with pH and temperature parallels quite closely the dissociation curves for HOCl so that all available evidence points to HOCl being the active agent in the disinfecting process In general levels of 0 5 to 1 0 p p m total residual chlorine are considered adequate for disinfection of water of good quality Where the water contains appreciable levels of organic materials the initial dose of chlorine must be increased to maintain the residual at the level indicated

HEAVY METALS Heavy metal disinfectants and antiseptics fall into three general categories mercury silver and a miscellaneous group having only feeble activity notably copper lead and zinc The last named are widely used as astringents due to their ability to precipitate protein and they appear to exert a favorable effect in promotion of wound healing Actual measurement of their bactericidal activity indicates that levels of 1 to 10 Gm /100 ml are required to kill many pathogens (Salle 1957)

Mercuric compounds both inorganic and organic find wide use in industry agriculture and medicine Mercuric chloride is widely used as a fungicide in both industry and agriculture but its use in medicine is quite limited due to its high acute toxicity The organic mercurial compounds have a much lower acute toxicity and have replaced the inorganic salts for most applications in medicine Examples of these compounds are *Mercurochrome Merthiolate and the phenyl* mercuric salts The first two are widely used for antisepsis the second and the third are used for preservatives in biologic materials intended for injection Appropriate concentrations of the latter two are 1 10 000 to 1 30 000

The mode of action of mercuric salts is probably the best known of any disinfectant *The Hg^{++} ion has been shown to interfere* with the metabolism of sulfhydryl compounds an effect which can be counteracted by supplying an excess of these compounds preferably in the form of glutathione although cysteine thioglycollic acid and inorganic sulfides also function as antagonists The combination of the Hg^{++} ion with the bacterial cell is apparently a rather loose one since it is easily displaced by competitors or removed either by precipitation as HgS or by adsorption onto activated charcoal Both vegetative cells and spores of fungi are susceptible to the action of Hg^{++} but bacterial spores are relatively insensitive While there is little doubt that mercuric salts are bactericidal in high concentrations there is no question that they are only bacteriostatic in low concentrations because of the ease with which their inhibition can be neutralized Because this system has been an important model for analysis of other disinfectants the reviews by Fildes (1940) and Rahn (1945) are of interest

Silver salts and silver metal have bactericidal properties even more marked than those of mercuric salts Although it appears that only Ag^{+} is active as a disinfectant and that Ag metal is active only through ionization little is known of the specific chemical nature of the lethal injury to the microbial cell Silver has been proposed for use in disinfection of water supplies and swimming pools but has found little use in this area Probably the most common use of silver salts has been the use of $AgNO_3$ in the prophylaxis of gonococcal conjunctivitis Silver proteinates also achieved a period of popularity in the treatment of conjunctivitis and gonorrhea but have been almost totally replaced by antibiotics

OZONE This pleasant smelling but irritating gas can be demonstrated to be a highly effective disinfectant under conditions of low *humidity in* laboratory investigations Unfortunately its activity is limited almost to

reproductive mechanisms of the cell, probably by interfering with a chemical oxidation basic to its growth Only the ionized form of the acridine is effective in bacteriostasis so that to be effective the molecule must be ionized as a cation to at least 50 per cent at pH 7 3 and 37° C (Sykes 1958) The subject of the acridine dyes has been thoroughly reviewed by Albert (1960)

Both categories of dyes have found limited use in medicine as bacteriostatic agents in burns and wounds They are well tolerated by the tissues although troublesome in regard to staining of linens In present practice they have been largely superseded by the sulfonamides and the antibiotics

CATIONIC QUATERNARY AMMONIUM COMPOUNDS This class of disinfectants and antiseptics is comprised of substituted amines of the general formula $R_1R\,R_3R_4N{-}X$ where R_1 through R_4 represent alkyl or heterocyclic groups and X an ionizable negatively charged group such as chloride or sulfate They are characterized by polar orientation at aqueous interfaces and apparently function as disinfectants by increasing the permeability of cell walls (Stedman *et al* 1957) Obviously the variety of aliphatic radicals permits a large number of variations on the basic structure which have been thoroughly exploited by commercial firms In distinction to the anionic detergents and the soaps the quaternary ammonium compounds are almost as effective against most gram negative as against gram positive organisms and for this reason have found increasing application in the past several decades They are effective against most vegetative forms of bacteria moderately effective against fungi and of limited activity against viruses and spores Their antimicrobial action is in many situations enhanced by the surface cleaning action resulting from their detergent properties

One distinctive characteristic of the cationic disinfectants is that they are almost completely inactivated by anionic detergents and soaps As a result particular care must be taken to remove all soap from the skin surface if one of the quaternaries is chosen for use as a skin antiseptic The same comment is applicable to surgical scrubs which are usually started with soap An intermediate rinse and scrub with 50 per cent ethyl alcohol is indicated if final washing and donning of gloves involves the cationic quaternary ammonium compounds

If this limitation is recognized, these compounds are excellent skin antiseptics and have found wide use in medical practice Another application of this group of compounds is in restaurant sanitation The choice of an appropriate detergent for use in sequence with a quaternary compound is critical as indicated above soap is undesirable

HALOGENS Of the halogens only two chlorine and iodine have importance as disinfectants The familiar tincture of iodine long used in medical practice has now been largely discarded in favor of less irritating or painful antiseptics The disinfecting action of iodine is apparently due to direct action of the molecular iodine on the surface of the microbial cell One class of iodine disinfectant has come into prominence within the past decade the iodophors or combination of iodine and a solubilizing agent or carrier These materials are characterized by a slow and prolonged release of iodine allegedly with far less irritation than is observed with the common tincture of iodine (Gershenfeld *1957)*

Chlorine has had a comparatively long and colorful history in the disinfection of public water supplies, and has probably contributed more than any other factor to the safety of water supplies Chlorine in gaseous form reacts with water to form hypochlorous acid HOCl through which chlorine exerts its antimicrobial action Chlorine is released from NaOCl available in grocery outlets the country over in the form of Clorox Other sources of chlorine include the inorganic chloramines in which chlorine substitutes for one or more of the hydrogen atoms in ammonia and the organic chloramines in which chlorine is substituted for one or more hydrogen atoms in an organic amine e g Chloramine T and Halazone All of the chloramines react slowly with water to release HOCl There is an important distinction in this regard between free chlorine (as HOCl) and total residual chlorine (which includes both HOCl and the chloramines) The two measurements differ because not all of the chloramine chlorine is immediately available

to the control of the dissemination of specific infectious diseases are excellently illustrated in the handbook *Control of Communicable Diseases of Man* (American Public Health Association 1965)

IMMUNIZATION

The term *immunization* is applied to two quite different procedures—active immunization and passive immunization *Active immunization* is the procedure whereby immunity is induced by the introduction of an antigen—ordinarily from an infectious agent—into the host to be immunized This process when successful induces a specific protective reaction in the host which resembles to a varying extent, the reaction induced by the disease itself After this response has been initiated the host retains the specific imprint of this stimulus for a relatively long time and can generally react vigorously and rapidly to the same stimulus should it recur in the future

Passive immunization on the other hand consists of the transfer of serum from an immunized or naturally immune animal (or man) to a susceptible individual Its usefulness results primarily from its relatively rapid action However its success depends on the presence of an effective titer of protective antibodies in the serum of the donor and is limited to those infectious diseases for which it can be shown that serum antibodies have a significant protective action Since many diseases (e g measles) are followed by a more or less lifelong antibody response the use of normal adult human serum for passive immunization against certain of the common childhood diseases is feasible

Passive immunization gives only a transient protection and the level of this protection falls as rapidly as the transferred immune serum is metabolized and (in the case of a heterospecific serum) eliminated immunologically as a foreign substance

Active Immunization

From the strictly immunologic point of view the introduction of any antigenic substance (e g albumin diphtheria toxoid bovine gamma globulin tobacco mosaic virus) by an effective route will lead in any normal animal to a state of immunization against the substance introduced In the case of inert antigens such as those just cited the response can be detected by demonstrating that the serum of the immunized animal can take part in such specific immunologic reactions as precipitation complement fixation antigen elimination or various other familiar immunologic reactions In many cases the animal not only exhibits a specific serologic response but will also acquire specific delayed type hypersensitivity to the antigen Many other manifestations of the specific immune response could be cited However when an infectious agent or a derivative of such an agent is used as the antigen, an additional series of phenomena in the immunized host may appear reflecting a specifically induced capability of killing or inactivating the infectious microbe involved The classic example is *Vibrio cholerae* infection Pfeiffer and Issaeff (1894) showed that when cholera vibrios were mixed with a specific anticholera immune serum and the mixture was injected intraperitoneally into a guinea pig the vibrios rapidly lost their motility became swollen and soon underwent lysis If a different vibrio species was used or if a normal serum was employed the vibrios remained actively motile and retained their normal morphology In a more direct type of experiment, it has been shown that fatal bacteremia—induced readily in normal rabbits by the intravenous injection of virulent pneumococci—is totally suppressed in animals previously inoculated with killed cultures of the same organism (Wright 1927) Such observations provide more direct evidence than does a physical reaction such as precipitation or agglutination to support the assumption that a given immunization procedure actually has a protective effect

Immunization of man has been undertaken by the preparation of one of four types of immunizing agents

1 **Live Attenuated Vaccines** Pasteur s development of an attenuated chicken cholera vaccine through aging of the culture (Pasteur 1880) an anthrax vaccine by cultivation of the organism at high temperatures and a swine erysipelas vaccine by passage of the organism through rabbits served as illustrations of the variety of approaches that may lead to the successful development of a living attenuated strain which is safe and yet

tally to surface disinfection, and even a thin film of mucus is sufficient to protect a micro organism from its action For this reason it has found little application in air disinfection Claims have been made for its efficacy in food preservation but even this application is of limited value (Ingram and Barnes 1954)

HYDROGEN PEROXIDE H_2O in a 3 per cent solution has been a common item of household use for decades It is a fair dis infectant acting by oxidation but is readily and rapidly broken down by the enzyme catalase and by hemoglobin As commonly used *in cuts and abrasions it is of very little* value because of the rapidity of its decompo sition

PERMANGANATES Permanganates are highly effective oxidizing disinfectants and have been commonly used in the disinfection of water that contains little or no organic ma terial Where organic compounds are present their use is not advisable Reaction with mi crobial contaminants or with organic com pounds produces the inert brown MnO_2 *which is inactive*

QUARANTINE AND ISOLATION

The procedures designed to achieve steri lization or disinfection described above are—except for skin disinfection—applied to in animate objects rather than people Numer ous measures for the prevention of infection are applied directly to people either as individuals or in groups The oldest of these in all probability are isolation and quaran tine These two measures though different in concept and application are so closely re lated that they are best considered together

Isolation is a measure employed to re move a sick person from contact with the community on the assumption that this will prevent him from spreading his disease to susceptible persons It probably began in principle (as noted earlier) with the rejection of lepers from organized communities many centuries ago It reached its apex during the early part of the microbial era However with growing knowledge of the prevalence of the carrier state in many diseases and of the infectiousness of many *diseases during* the late incubation period the limitations in its value to the community have become ap parent It will continue to be employed—as a matter of good public health practice as well as wise public policy—for diseases such as smallpox, which are regarded as *major* threats to the community, but its use even in smallpox cases may in the future be limited to the time it takes to render a patient non infectious by appropriate chemotherapy For less serious diseases such as scarlet fever and diphtheria it may retain a useful place inso far as it can reduce the risk to highly sus ceptible individuals, such as younger siblings in a family situation

On the other hand isolation often has *very real value for the patient, especially in* those diseases such as measles which are prone to have serious respiratory complica tions However, it is important to emphasize that isolation in the interest of the patient may be applied with equal justification to in dividuals with certain noninfectious diseases —*e g* congestive failure or a leukemic re lapse—who not only require protection from the prevailing epidemic respiratory disease agents but probably are endangered even by contact *with* the ordinarily nonpathogenic flora in the nasopharynges of their friends and relations The difference between these two situations is one of detail not principle

Quarantine, a more recent measure of control is the segregation under isolated conditions of individuals suspected of har boring a communicable disease Its history is described vividly by Meyer (1962) Quarantine arose in an attempt to stem the spread of plague in Europe in the 14th cen tury and has been applied extensively since then to almost every known communicable disease Its effectiveness is clearly dependent on the infectious period of the disease the ability to identify correctly the persons who may be infected and the promptness and accuracy with which quarantine actions are taken Like isolation its routine use has be come progressively more and more limited However there are situations in which it can be employed in conjunction with reliable laboratory procedures or chemoprophylaxis with considerable efficiency as a means of minimizing the spread of a dangerous com municable disease As a practical example *such a combined approach* can be applied logically to cholera

The applications of these two approaches

group Some such as the capsular substances of pneumococcus types II III and VIII have been identified as relatively pure polysaccharides others such as that of pneumococcus type I have been shown to be polysaccharides containing a small proportion of amino groups Still others such as the so-called O antigens of the salmonella have been found to be complex macromolecules consisting of polysaccharides lipoids and proteins loosely united to form the basic structure of the bacterial cell wall especially of gram negative bacteria Lipopolysaccharides are difficult to characterize because they disaggregate on chemical and physical treatment and their antigenicity may be lost on purification

Pattern of the Immune Response

The response to the injection or the administration of an antigen will vary depending on a great many qualifying factors many of which have been discussed in a recent excellent review (Cluff and Allen 1962) With sufficiently sensitive systems antibodies may be detected in the serum of the inoculated animal as early as 24 hours after intravenous injection of the antigen (Uhr Finkelstein and Baumann 1962) in the course of a rapid increase in antibody level which is essentially logarithmic in character The peak of this response may be reached in about a week or 10 days Following this the level of antibodies at first falls almost as rapidly as it rose with a progressively slower rate of fall leading to an asymptotic level which decreases very slowly over many many weeks and occasionally persists at a measurable level for years If the injection is made subcutaneously the initial appearance of antibody will be delayed the peak may be reached somewhat later and the rate of fall may be more gradual A typical response in man is shown in Figure 2

In a number of instances it has been shown that the inoculation of polysaccharide antigens results in a different type of response the antibody rise comes on early and rapidly reaches a high level but the level is maintained without the rapid drop and the asymptotic leveling that generally has been found with most protein antigens Examples of such patterns are given by Burnet and Fenner (Fig 3) and in the detailed and quantitative

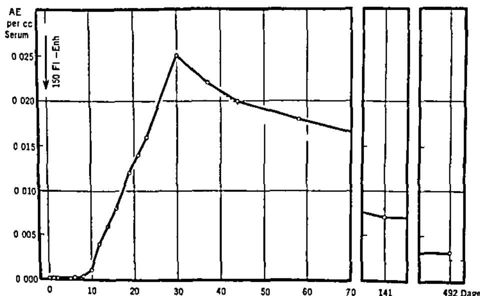


Fig 2 Effect of a *single* injection of 150 flocculating units of concentrated and purified anatoxin in a 4 year old child without antitoxin prior to the injection (From Jensen C 1933 Acta path microbiol scand *10* 137)

effective as a vaccine Although more sophisticated methods for discovery selection or creation of strains meeting the essential criteria for live vaccines now are available the development of an attenuated vaccine still depends as much on good fortune as it does on good experimental design

2 **Inactivated Vaccines** Killed suspensions of plague bacilli typhoid bacilli and cholera vibrios developed in the last decade of the 19th century were the first of many such preparations used in the immunization of man

3 **Antigen Antibody Mixtures** Besides numerous such preparations for veterinary use there has been one notable example—diphtheria toxin antitoxin mixture developed by various investigators and culminating in the studies of von Behring (1913)—which has been successfully employed in the past for the immunization of man However it requires skillful balancing of the proportions of toxin and antitoxin to produce a mixture which is antigenic yet not clinically toxic

4 **Colloidal or Fluid Derivatives of Bacterial Cultures** Filtrates or supernatants of bacterial cultures have been most effective as immunizing antigens when they contained exotoxins (e g diphtheria and tetanus toxins) which when rendered safe by detoxification could be used to produce immunity against the toxin The work of several earlier investigators pointed the way to Ramon s definitive observations (1924) on the use of formalin for detoxification of exotoxin-containing culture filtrates The resulting products (called *anatoxins* by Ramon and *toxoids* in the English and the German speaking countries) have been used effectively on a world wide scale

By grinding bacteria in a ball mill disrupting them with ultrasonic vibration freezing and thawing them or ejecting a bacterial suspension through a small orifice under high pressure, extraction and purification of bacterial antigens can be facilitated Many experimental antigens (but few vaccines for human use) have been prepared this way Such procedures are in most cases limited by difficulties in defining and chemically purifying the many antigens that are contained in any given microbial species Even where adequate technics for such purification and separation exist there is often no reliable information as to which particular antigen or antigens in the microbe are significant for protective immunization There are a few exceptions such as the anthrax bacillus from which a number of antigens have been characterized or demonstrated over a period of years and the actual protective antigen recently identified The best defined antigens are diphtheria and tetanus toxoids which have been highly purified by Pillemer and his associates (1946 1947) and various highly purified pneumococcus polysaccharides (see Chap 16) All of these have been well characterized physically and chemically and have been shown to be immunogenic against their respective diseases On the other hand none of the characterized antigens of the typhoid bacillus (see Chap 25) has been unequivocally shown to be associated with immunity to typhoid fever in man (Edsall et al 1959)

Antigens

The term *antigen* has been used loosely up to this point to refer to any substance or complex of substances capable of inducing an immune response However purified well characterized antigens can be divided into several chemical categories and the antigenic behavior of each of these categories appears to depend in considerable part on its chemical nature The majority of the well studied antigens are proteins or protein complexes Many fairly well characterized and purified proteins—e g egg albumin bovine serum albumin hemocyanin—have been used for experimental studies on the immune response From the point of view of infectious disease control only a few protein antigens known to induce protection against disease have been prepared and purified—chiefly the formalin inactivated toxoids derived from diphtheria and tetanus toxins and certain other bacterial exotoxins These are proteins with a molecular weight in the neighborhood of 70 000 which have the fortunate property of retaining their ability to stimulate resistance and produce antibodies after they have been detoxified e g with small amounts of formalin

Another very large class of effective and significant antigens is the polysaccharide

in the inoculation of typhoid bacilli in rabbits was similar to the Freundlich adsorption isotherm equation In the modified expression of this equation $Kc^{1/n} = Ab$ K and n are constants c is the concentration of antigen and Ab is the maximum concentration of antibody obtained Graphically the dose response relationship is expressed in terms of a straight line with a slope of $1/n$ For some antigens n is a relatively small quantity and therefore the slope is relatively steep whereas for others n is a much larger quantity and the slope is relatively flat. The range of n values interpolated from the literature by Stevens runs from 0 6 for diphtheria toxoid as a primary stimulus in the guinea pig to 3 6 for Vi antigen in the mouse No very clear-cut generalization appears to be available for these data their major significance is that the dose response relationship appears to vary considerably depending on the antigen and probably also on the host,

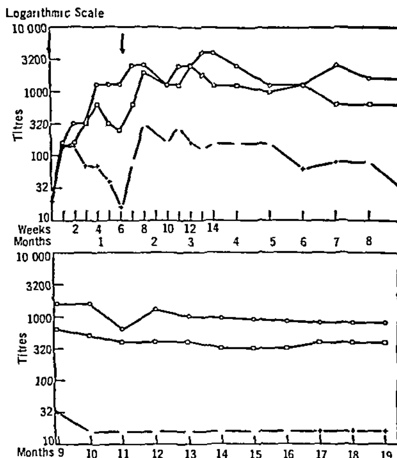


FIG 4 Agglutinin titers (mean) in rabbits after subcutaneous injections of formalin killed typhoid bacilli. A +—+ suspended in salt solution B □—□ combined with Aquaphor and liquid petrolatum. C ○—○ combined with Aquaphor and liquid petrolatum containing killed tubercle bacilli ↓ injection (From Freund J and Bonanto M V 1944 J Immun 48 325 334)

studies of Heidelberger *et al* (1946) on the serologic response of man to the inoculation of pneumococcus polysaccharides In the latter studies the antibody levels remained relatively constant for 5 to 8 months and tapered off only gradually during a period of 2 years or so One explanation offered for this pattern of response is that polysaccharide antigens are frequently more resistant to the natural digestive enzymes of the mammalian host than are most of the protein antigens hence they persist for a longer time and maintain their antigenic stimulus accordingly

The physical state of the antigen which is administered appears to make a significant difference in the response Antigens may in essence be either soluble (e g fluid diphtheria or tetanus toxoid) or particulate (e g aluminum hydroxide absorbed toxoids whole bacterial antigens virus suspensions etc) In a systematic comparative study with diphtheria toxoid Freund and Bonanto (1941) showed that the aluminum combined antigen was more effective when given intravenously than when given subcutaneously and that the reverse was the case for the fluid antigen Thus no single characteristic of the antigen or its administration can be considered by itself but interactions appear to prevail between particle state route of administration etc

Route The mechanisms underlying the influence of the route of administration of an antigen have not been adequately studied partly because of the many variables involved The intravenous route is used widely in animal studies because it appears to induce the most rapid massive and reproducible response However subcutaneous or intramuscular inoculation usually is employed in human immunization not only because of its simplicity but also because it reduces the absorption rate and hence the risk of acute reactions

It is widely believed that inoculating an antigen intracutaneously will enhance its effectiveness However studies on the use of tetanus toxoid by this route versus the subcutaneous route (Barr Sayers and Stamm 1959) suggest that the influence of route in this instance is minimal In general the effect of delayed absorption of antigen appears to be beneficial in regard to duration of the antibody response and suppressive in regard to the height of the immediate response

Dose The relation of antigen dose to the antibody response has frequently been validated although there is no simple generalization on this point Stevens (1956) has revived and extended an ingenious observation of Smith and St John Brooks (1912) who showed that the dose response relationship

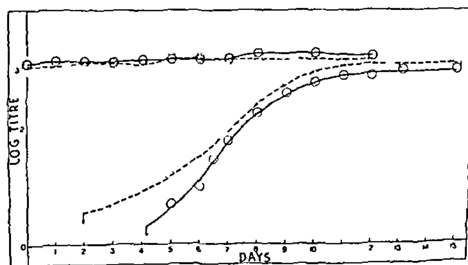


FIG 3 Primary and secondary response of two rabbits given rickettsial emulsion intravenously Note absence of secondary response (From Burnet F M and Fenner F 1949 The Production of Antibodies p 17, Melbourne Macmillan)

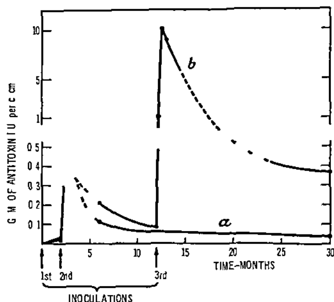


FIG 5 Tetanus antitoxin titers in the blood of persons receiving (a) two doses and (b) a third dose of fluid tetanus toxoid (From Evans D G 1943 Lancet 2 316 317)

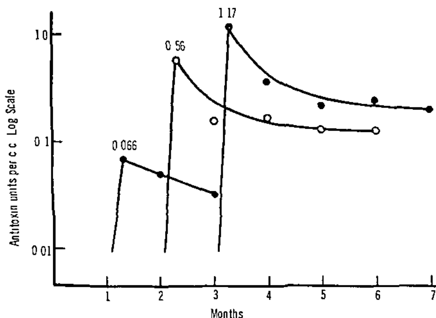


FIG 6 Geometric mean serum antitoxin levels in 3 groups of 15 guinea pigs given 2 doses of 0 25 Lf each of alum precipitated toxoid at intervals of 1 2 or 3 months respectively (from Barr M and Glenny A T 1945 Some practical applications of immunological principles J Hyg *44* 135 142)

the route of administration and other factors

Adjuvants Many substances have been shown to enhance the response produced by a given amount of antigen Major emphasis has been focused upon two types of adjuvant procedures The oldest is adsorption of the antigen onto aluminum salts (e g alum aluminum hydroxide aluminum phosphate) More recently water in-oil emulsions of antigens prepared with purified mineral oils and using a variety of emulsifying agents to produce an effective stable mixture have been widely employed Various aluminum salts have been shown to provide a marked degree of enhancement of the antibody response especially for bacterial toxoids and bacterial vaccines Their value for polysaccharide antigens has not been so clearly defined A number of technical factors such as an aluminum salt concentration pH etc bear on their effectiveness (Levine Stone and Wyman 1955) and the degree of this enhancing effect varies with different animal species (Ipsen 1954b) The water-in-oil emulsion type of adjuvant first described in detail by Freund and Bonanto (1944) has been employed very extensively in a great many studies of the immune response in animals and on a more limited scale in studies in man It produces extraordinary enhancement in the peak antibody level obtained and it leads to a remarkably sustained antibody response which may last with little diminution for several years (Fig 4) The exact nature of the mechanism of its enhancing effect is not known It has been clearly shown (Freund and Lipton 1955) that it is not due to retention of the antigen at the site of inoculation since early excision of the antigen site does not interfere with the effect It is generally believed that it leads to relatively prompt and rather widespread deposition of antigen in antibody forming tissues and ready uptake by the appropriate cell system for antibody production Interestingly it is relatively ineffective in the enhancement of the response to polysaccharide antigens

It has long been known (Maclean and Holt 1940) that the mixing of a bacterial vaccine with a toxoid could result in an enhanced antigenic response to the toxoid More recently, it has been shown (Johnson, Gaines and Landy 1956) that bacterial cell wall lipopolysaccharides or endotoxins have an enhancing effect on protein antigens The mechanism of action of endotoxins is different from that of adsorbing or emulsifying adjuvants in that the endotoxin effect can be obtained without the necessity of mixing the endotoxin with the antigen

A number of other adjuvant substances have been reported to be effective and some new ones are under study their general application range of effectiveness and mechanism of action have not yet been determined

The *persistence of measurable antibody* after a primary stimulus of antigen varies enormously, and it is evident that this variation is due only in part to the variation in sensitivity of methods for detecting antibody The sustained high levels of antibody following the inoculation of various polysaccharides have been noted above The administration of live attenuated vaccines may lead to a massive release of antigen This can produce a marked primary peak response following the subclinical infection that they produce with a subsequent fall leading to an asymptote at a low level not unlike that described for simple protein antigens above The antibody response to live measles vaccine is an excellent example (Krugman 1962) On the other hand the pattern of the primary immune response to most nonviable antigens may be difficult to determine because antibody levels frequently fall below measurable amounts within a relatively short period of time

The Secondary Response

In the case of almost all antigenic stimuli except those which produce a high and sustained initial antibody level the reintroduction of the same antigen after a period of time (the optimal interval varies with the antigen the animal species and other factors) will result in a more rapid and in many cases much higher rise in antibody level than was seen following the primary stimulus The rapidity of the secondary antibody rise is generally thought to be greater than that of the primary stimulus However in careful quantitative studies of one particular antigen antibody system Uhr *et al* (1962) found the rate of increase in serum antibody titer

mune response Marked differences have been observed in the immune response in different animal strains (Ipsen 1954a) Ipsen has also demonstrated differences in response related to the environmental temperature at which the animals were held (Ipsen 1952) Several reports of a suppressive effect of antibiotics on development of resistance have appeared but only a few have distinguished between the immunizing effect of infection and the effect of inoculating an inert antigen (e g Stevens 1953) Various investigators have reported that salicylates may interfere with one or more immunologic systems Wilkens and Tasman (1959) found a markedly reduced response to tetanus toxoid in tuberculous patients by contrast the *response in patients with chronic hepatic disease* which might well be expected to be impaired has been found actually to be enhanced (Havens Shaffer and Hopke 1951)

Innumerable studies have been reported on the effect of nutrition on the immune response It has been reported that the antibody response in man is essentially unaltered even in the presence of severe nutritional deficiencies (Balch 1950) On the other hand there are massive data to indicate that there is an intimate relationship between the nutritional status and susceptibility to a variety of infections (e g Dubos and Schaedler 1959 Scrimshaw Taylor and Gordon 1959) These findings serve to emphasize the fact that antibody responses are by no means the only factor of significance in determining resistance to infection

Limitations of Immunization

In spite of the variety of ways in which immune responses may be measured little is known in many cases concerning the interrelation between the antibody response produced by a particular antigen and the protection obtained against the disease in question A classic example is typhoid fever The most readily isolated antigen from the typhoid bacillus is the Vi antigen which can be prepared in a relatively purified state It produces striking antibody responses in rabbits and is a potent protective antigen for mice exposed to subsequent challenge with typhoid bacilli (Landy and Webster 1952) Yet the field studies on the protection of man with typhoid vaccines (Yugoslav Typhoid Commission 1962) failed to provide any significant evidence that the Vi antigen is important in man

The degree of protection achieved by different immunizing procedures varies enormously Tetanus toxoid effectively used leads to nearly 100 per cent protection as does yellow fever vaccine Typhoid vaccine may give approximately 75 per cent protection (Yugoslav Typhoid Commission 1962) or better On the other hand immunization against such diseases as brucellosis even with live vaccines has yielded relatively inconclusive results

The reasons for the great differences observed in the effectiveness of immunizing procedures are various and frequently not known *It is believed that in such diseases* as brucellosis the intracellular persistence of infection in receptive tissues renders it almost impossible to eradicate infection hence subsequent relapses may occur in such cases It appears that antibodies in the circulation are (for unexplained reasons) ineffective in suppressing such relapses Furthermore it has been clearly shown that relapses may occur in typhoid fever even in the presence of high circulating antibody levels

Indeed in a number of instances the relation of immunity to antibody levels is unclear Perhaps the most striking is tuberculosis in which only limited evidence exists to date indicating any relationship between the two Yet immunization with the live attenuated tuberculosis vaccine (BCG vaccine) has been thoroughly demonstrated in well controlled studies to produce a marked diminution in the incidence of tuberculosis in the vaccinated (e g Medical Research Council 1963) In the case of tuberculosis and certain other infections it has been suggested that immunity may be related more closely to the development of a delayed allergic response than to the development of serologic resistance Such findings are further enhanced by the observation that a number of illnesses particularly the viral exanthems can produce lasting immunity in patients with agammaglobulinemia even though no measurable antibodies can be demonstrated in such patients even in convalescence

Neonatal Immunization

A number of studies have shown that im

to be very similar in both primary and secondary responses The peak level may be achieved in about 8 to 10 days following the secondary stimulus and an almost equally rapid initial fall in the antibody level may ensue However in general the asymptotic level reached after a secondary stimulus is higher than was the case after the *primary* stimulus Thus repeated inoculations properly spaced frequently may be employed to achieve successively higher and higher residual persisting antibody levels

A typical illustration of the general pattern that is observed most frequently in successive primary and secondary responses is shown in Figure 5 Here it is seen that the *initial inoculation of a fluid tetanus toxoid* produced an extremely small response which was still increasing at the time of the second dose 2 months later A vigorous response to the second dose occurred followed by a sharp fall to a low asymptote with a residual level which was still easily detected after 1 year At this time one half the subjects were given a third dose As shown in the figure their subsequent titer leveled off at a point much higher than that achieved by those who had only two doses On the other hand where the primary response is sustained at a high level the response to a second dose may be minimal or undetectable (see Fig 3) This is seen most commonly with polysaccharide antigens

Interval Between Antigenic Stimuli The capacity to induce a clear-cut recall or booster response appears to depend on an adequate interval between the first inoculation and the next one Together with clinical impressions and a few isolated studies in man an enhancing effect of increase in interval has been demonstrated in a few animal studies (Fig 6) Coons and Fecsik (1960) using tetanus toxoid in mice have shown that the enhancement of the response to the second dose of toxoid increased progressively up to an interval of about 21 days leveled off for the next 140 days and showed no sign of diminishing Clearly some sort of latent period is required after the primary antigenic stimulus for the development of the capacity to respond to a recall dose

Whatever may be the mechanism the secondary response is *probably the* result of a stimulus which apparently leads to a very rapid multiplication of the specifically oriented antibody forming cells This multiplication of cells was found to proceed at a rate of about one replication every 7 hours through 8 cycles after which it came to a halt (LeBlond and Ste Marie 1960) Thus over a period of about 3 to 4 days the capacity *to form a particular antibody may be* increased on the order of 100 fold Subsequent increases in the antibody concentration may be considered as due primarily to the steady accumulation of antibody put out by the increased number of antibody forming cells which for a while overbalances the steady catabolic decrease of homologous protein in the host It is also possible that a certain amount of preformed antibody is *liberated* from the specific antibody forming cell at the time of the secondary stimulus

Several observations suggest that the *dose response relationship following the secondary stimulus* may be quite different from the dose response relationship after the primary stimulus In a study by Ipsen (1953) in man the dose response slope was much flatter after a second dose of tetanus toxoid than it was for the first injection It is yet not known whether this is a general phenomenon

The duration of the antibody response after successive inoculations has in some cases been shown to be remarkably long lasting as is also *the capacity to respond* to a very long delayed booster inoculation Both diphtheria and tetanus antitoxin levels have been shown to persist for years after basic immunization and one reinforcing dose Various recent studies have demonstrated that measurable tetanus antitoxin levels may persist from 7 to 22 years after the last preceding inoculation and that rapid rises in titer will occur in such individuals following a booster inoculation In one such study Gottlieb *et al* (1964) derived a mathematical formula for expressing the level of residual antitoxin and of postbooster peak antitoxin levels Their equation predicts a postbooster rise not unlike that forecast by the cellular observations of LeBlond and Ste Marie (1960) It also indicates that at least traces of antitoxin (although not necessarily at a measurable level) probably persist for a lifetime after adequate tetanus immunization

Host Factors Many host factors also influence the nature and the degree of the im

catabolic process continues indefinitely at a logarithmically uniform pace characteristic of the host species involved and perhaps also of the protein injected Third if the protein injected is antigenically heterologous it will act as an antigen and stimulate the formation of antibodies against itself As these antibodies enter the circulation they will combine with the injected protein and thus accelerate its disappearance from the circulation These three phases of the declining level of intravenously administered antibodies are illustrated in Figure 7 which shows the level of both homologous (rabbit) gamma globulin and heterologous (bovine) gamma globulin injected into a rabbit It is seen that in this example both follow essentially the same pattern of elimination through the fourth day Thereafter the level of the heterologous (bovine) gamma globulin falls more rapidly due to the immune reaction which its injection has engendered

Numerous studies on the half life of injected homologous or heterologous gamma globulins have yielded varied and sometimes apparently conflicting data due to the many physiologic and immunologic variables involved However certain generalizations can be made in regard to the sequence of events following the introduction of antibodies into man Human gamma globulin exhibits a half life of about 30 days in the infant and perhaps less in the older child or the adult Heterologous antibodies (e g equine tetanus antitoxin) appear to be metabolized more rapidly especially if they have been modified by enzymatic digestion (McComb and Dwyer 1963) As in the case of bovine gamma globulin shown in Figure 7 heterologous sera will be more rapidly eliminated as soon as specific antibodies appear in the host in response to their injection In persons who have been injected previously with a heterologous serum another injection of serum of the same animal species will serve as a booster injection which usually leads to more rapid elimination Thus in some individuals the half life of a foreign serum to which they are already sensitized may be as short as 1 or 2 days

If the serum is injected intramuscularly or subcutaneously its distribution into the circulation (and hence into the extravascular space) is markedly delayed the peak titer of antibodies thus injected into man will not be reached for 48 to 72 hours

Applications In practice passive immunization has been most widely and most successfully used in the control of those diseases which injure the host primarily by the liberation of a toxic substance Thus the administration of 1 000 to 1 500 units of the appropriate equine antitoxin apparently has been quite effective in the prevention of clinical diphtheria or tetanus respectively in exposed human beings Although few antibacterial serums have been found useful in prophylaxis an exception is antipertussis serum which has been found to be highly effective in the prophylaxis of this disease in exposed susceptible infants On the other hand many serums both antitoxic and antibacterial have been widely accepted at first only to be shown later to have little if any value The ineffectiveness of passive immunization in many cases may be due to a number of factors The antibodies administered are often given too late or the titer of the preparation used may be too low or serum antibodies may simply play a minor role in prevention or cure of the disease in question

Various forms of human serum or its derivatives have been used in prophylaxis of several human viral diseases The classic example is measles for which pooled normal adult human serum convalescent serum pools of the gamma globulin fraction from human placentas (which are rich in adult serum) or the gamma globulin fraction of pooled adult plasma have all proved to be highly effective in the prevention or the modification of measles This is because the vast majority of adults in most communities have had measles because measles antibodies persist for a long time after recovery from the infection and because a small amount of measles antibody appears to be effective in prophylaxis Human gamma globulin is also effective in the prophylaxis of infectious hepatitis but there are few other diseases for which gamma globulin has been shown clearly to have prophylactic value

The preparation of a gamma globulin concentrate from pooled human serum has two advantages over the use of unconcentrated

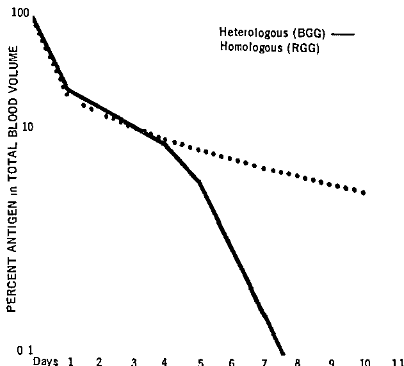


FIG 7 Blood levels of rabbit gamma globulin and bovine gamma globulin injected intravenously into rabbits (From Dixon F J et al Fate of I^{131} labelled bovine gamma globulin in rabbits *in* Pappenheimer A M Jr (ed) 1953 Nature and Significance of the Antibody Response p 172 New York Columbia University Press)

munization of very young infants frequently is relatively ineffective compared with the results obtained in older infants or young children In many cases this is explained simply by the fact that such infants possess antibodies transferred from the maternal to the fetal circulation and such antibodies will repress the response to newly introduced antigens Cooke (1948) demonstrated this effect clearly by the response to combined diphtheria and tetanus toxoids in young infants None of the infants possessed maternally derived tetanus antitoxin and all responded well to the tetanus toxoid component A number of the infants had relatively high levels of maternally transmitted diphtheria antitoxin these responded poorly to the diphtheria toxoid component whereas those infants that did not have a significant level of diphtheria antitoxin responded well Many other studies indicate that in general the young infant will respond well to immunizing antigens but its response may be blanketed or suppressed by the presence of maternal antibodies

PASSIVE IMMUNIZATION

Passive immunization may be carried out with serum derived from the same species as the recipient or with serum from another species It may be based on the injection of serum containing naturally acquired antibodies serum from convalescent persons or serum from persons or animals hyperimmunized against the disease in question Crude serums are rarely used nowadays most preparations being chemically processed (fractionated) to yield a relatively purified concentrate of the antibody-containing gamma globulins However for convenience and brevity the term serum will be used in this section in discussion of characteristics common to all preparations processed or unprocessed

Pattern of Response The intravenous injection of a dose of antibody containing serum leads promptly to a maximal circulating titer of the antibody or antibodies present This level starts to fall immediately for at least two—and in certain cases three—reasons First the serum proteins introduced into the circulation will inevitably be redistributed in the extravascular fluid spaces of the recipient, and second the recipient commences at once to metabolize the introduced protein whether it is antigenically homologous or not The redistribution process is completed in a day or two but the

from 30 per cent to zero within 2 weeks by administering 6 to 8 Gm of sulfadiazine to every person in the group Following use of the higher dose of drug the carrier rate subsequently rose to only 5 per cent 12 weeks later Smaller doses of the drug led to a smaller decrease in the carrier rate and to a more rapid rise toward the preceding rate Mass use of sulfadiazine in the face of major epidemics of meningococcus meningitis probably has been employed more extensively in the Sudan Nigeria and neighboring countries in subtropical Africa than anywhere else for it is during the dry cold harsh winters of the semiarid areas of this region that the worst epidemics of this disease on record have occurred (LaPeysonnie 1963) Of course the effectiveness of such a chemoprophylactic procedure depends on the continued dominance of relatively drug susceptible strains of the organism in the community when relatively resistant strains appear chemoprophylaxis may be unsuccessful (Millar *et al* 1963)

Prophylaxis of tuberculosis with isoniazid has become a well established procedure adaptable to individual or community use In a large study in the United States 12 500 household contacts of active cases of tuberculosis were given isoniazid and a comparable group received a placebo in a double blind study Medication was maintained for 1 year in most cases Ninety four cases of tuberculosis developed in the control group during the year of the study as compared with 23 in the group taking isoniazid (Ferebee and Mount 1962)

In practice chemoprophylaxis directed against tuberculous infection may be applied either (as in the study cited) to all persons suspected of having been infected or only to individuals with a recently acquired positive tuberculin test In the latter case the procedure involved is literally speaking chemotherapy of subclinical infection

Sometimes chemoprophylaxis of one disease is accomplished by the chemotherapy of another The best example is rheumatic fever which occurs as a late complication in a small percentage of β hemolytic streptococcal infections Early and effective chemotherapy of such infections with sulfadiazine or penicillin greatly reduces the incidence of rheumatic fever However unless the infecting streptococcus is eliminated from the patient, the attack rate of rheumatic fever is not significantly reduced (Catanzaro Rammelkamp and Chamovitz, 1958)

Since individuals who have had one attack of rheumatic fever are prone to have repeated attacks genuine chemoprophylaxis i e prevention of the occurrence of streptococcal infections is widely used in such individuals who may receive the drug regularly for as long as 5 years The long duration of such a prophylactic program designed to avert *repeated* infections with a rapidly multiplying organism is different in concept from the long term prophylactic regimen for suspected or incipient tuberculous infection which is aimed at a single infection caused by an organism having a long generation time

Chemoprophylaxis is employed effectively for several other categories of microbial infections—e g to prevent the development of endocarditis in patients with congenital or acquired cardiac defects to prevent the development of meningitis from nasopharyngeal bacteria as a complication of skull fracture or to prevent congenital syphilis in an infant carried by a pregnant syphilitic Chemoprophylaxis has been used in the control or the prevention of recurring or chronic pyelonephritis However its value in the latter situation is limited because drug resistant organisms tend to replace the initial infecting organism for which the chemoprophylactic agent was selected Indeed the frequency of such superinfections (see Chap 40) is the primary reason that prolonged sulfonamide or antibiotic prophylaxis with only a few exceptions has failed to control secondary or complicating infections in the respiratory tract recurring infections of the genitourinary tract infections following surgical operations severe burns etc In most such situations there are numerous microorganisms present which can multiply in the presence of the antibiotic used, and which will do so with vigor when other competing organisms have been suppressed

Successful chemoprophylaxis is by no means limited to bacterial infections Aside

serum Fractionation concentrates the antibodies in the serum roughly 15 to 30 times and also it appears (for reasons not fully understood) to eliminate the hazard of serum hepatitis which is a significant risk with unfractionated human serums

For the reasons outlined above the effectiveness of passive immunization is transient and the individual injected will after a short period generally be as susceptible to the disease in question as he was before the inoculation An exception arises in the case of what is called *passive-active immunization* This may occur in some instances when the individual is infected with the disease agent in question during the time that he has a small amount of the corresponding passively acquired antibody in his circulation For example injection of a relatively small dose of gamma globulin into a child exposed to measles will usually lead to what is called modified measles —a mild form of the disease which generally results in lasting immunity Likewise individuals exposed to infectious hepatitis and given a small dose of gamma globulin may develop subclinical hepatitis followed by demonstrable immunity However, proved examples of such passive active immunization are relatively few and the difficulties of establishing evidence for the occurrence of such a process are apparent

Newborn infants of many mammalian species acquire passive immunity from the mother through antibodies transferred via either the placenta or the colostrum This passive immunity like any other homologous passive immunity wanes with time according to a definite and determinable half life It serves to protect many infants from a variety of diseases to which they would otherwise be extremely susceptible but it leaves them highly susceptible during the period between the disappearance of maternally transferred antibodies and the development of active immunity from natural or artificial means However in some instances it doubtless provides a basis for passive active immunization an example of which is the occasional development of immunity to vaccinia virus without demonstrable vaccinia lesions (Kempe and Benenson 1953)

The application of maternally transmitted passive immunity in the prevention of infectious diseases of infants has been approached by immunizing mothers prior to delivery of their infants Clear-cut evidence for the effectiveness of such a procedure has been obtained by Schofield *et al* (1961) in the case of neonatal tetanus a disease of major importance in many parts of the world The principle has also been employed—with less evidence for its efficacy—for protection of infants against neonatal diphtheria and pertussis

CHEMOPROPHYLAXIS

Although the specific prophylactic properties of cinchona bark against malarious fevers have been known for several centuries and the merits of mercury or silver salts in the prevention of venereal disease likewise have long been recognized specific chemoprophylaxis was essentially empiric and limited in scope until recently However, the sulfonamides the various antibiotics and certain other recently developed drugs have provided a basis for the effective development and application of chemoprophylaxis against a number of infectious diseases

Individual chemoprophylaxis may be directed at preventing the initiation of infection at suppression of an infection during the incubation period or at aborting an established infection before it progresses to a more serious phase or to complications In addition chemoprophylaxis applied to groups of people may be useful in reducing the prevalence of the organism concerned—thus reducing the frequency of its spread from person to person and hence preventing or aborting an epidemic situation

An outstanding example of the last mentioned application of chemoprophylaxis is the administration of sulfonamides—notably sulfadiazine—to suppress outbreaks of meningococcus meningitis Such outbreaks occur characteristically in large, crowded military recruit populations among whom the carrier rates for a given type of *Neisseria meningitidis* may at times exceed 30 per cent (Aycock and Mueller 1946) These investigators showed that the carrier rate could be reduced

from 30 per cent to zero within 2 weeks by administering 6 to 8 Gm of sulfadiazine to every person in the group Following use of the higher dose of drug the carrier rate subsequently rose to only 5 per cent 12 weeks later Smaller doses of the drug led to a smaller decrease in the carrier rate and to a more rapid rise toward the preceding rate Mass use of sulfadiazine in the face of major epidemics of meningococcus meningitis probably has been employed more extensively in the Sudan Nigeria and neighboring countries in subtropical Africa than anywhere else for it is during the dry cold harsh winters of the semiarid areas of this region that the worst epidemics of this disease on record have occurred (LaPeysonnie 1963) Of course the effectiveness of such a chemoprophylactic procedure depends on the continued dominance of relatively drug susceptible strains of the organism in the community when relatively resistant strains appear chemoprophylaxis may be unsuccessful (Millar *et al* 1963)

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from examples such as malaria noted above (for which far better prophylactics than quinine are now available) and Gambian trypanosomiasis (for which injection of a pentamidine salt will provide protection for up to 6 months) there are several other well defined applications In persons heavily exposed to scrub typhus a rickettsial disease early sustained administration of chloramphenicol during and for 2 weeks after exposure only postponed but did not prevent the onset of the disease On the other hand intermittent doses of the drug given at intervals of 4 to 7 days for 4 to 6 weeks provided almost complete protection (Bailey and Ley 1952) The failure of early sustained chemoprophylaxis was attributed to the rickettsiostatic effect of the drug which suppressed the antigenic stimulus of full blown infection but did not entirely eradicate the infecting organism On the other hand intermittent prophylaxis permitted the latent infecting organism to replicate and liberate antigen but generally kept its replication below the level which induced clinical disease These findings illustrate another of the problems of chemoprophylaxis for the partial suppression of the normal immune response following early antibiotic therapy has been recognized as a problem in a number of infectious states

The pattern of chemoprophylaxis of Q fever with oxytetracycline has been described in detail by Tigertt and Benenson (1956) and it is to be expected that other rickettsial infections as well as infections with psittacosis and related viruses could be prevented similarly by the carefully planned use of antibiotics An unexpected new finding is the remarkable effectiveness of a derivative of thiosemicarbazone in preventing smallpox in exposed contacts (Bauer *et al* 1963)

Chemoprophylaxis has been employed widely on an empiric basis for a variety of conditions e g chronic or recurrent respiratory infections Generally such procedures are unsupported by evidence of their success and in some instances the ineffectiveness of such nonspecific shotgun chemoprophylaxis has been clearly documented (e g Petersdorf and Merchant 1959)

MICROBIAL INTERFERENCE

The possibility that the multiplication—or even the survival—of a pathogenic organism could be controlled by the growth of a harmless competitive microbe was first recognized by Pasteur in the 1870 s It has been given vivid specific meaning in the discovery of numerous antibiotics which are produced by one microorganism and are capable of inhibiting others The general concept of antibiosis as an ecologic phenomenon is discussed by Waksman (1947) However besides the identified antibiotic substances there are many instances in which the phenomenon of microbial interference has been recorded without a specific antibiotic substance being implicated For example certain strains of lactobacillus when ingested in large quantities will markedly alter the existing intestinal flora and in particular will reduce the numbers of coliform bacteria present Extensive studies on this phenomenon have been carried out by Rettger *et al* (1935)

On the other hand the numerous instances of superinfection (see Chap 40) which have resulted from the alteration of the pattern of the microbial flora of the respiratory the intestinal or other systems by vigorous or sustained antibiotic therapy provide evidence that, first there is a nicely maintained balance between the microorganisms normally present in and on the human body and second the eradication of only a few components of this complex system may permit other members of the system which normally are present only in small numbers to multiply vigorously and sometimes to become dominant in the local host parasite ecosystem

In spite of the attractiveness of the concept and the variety of observations which lend it plausibility there are surprisingly few instances in which it has been applied successfully A number of small scale observations on the implantation of lactobacillus (e g Hawley Shepherd and Wheater 1959) and the application of this technic for the control of various infections (e g Beck and Necheles 1961) have appeared in print in

recent years However sufficient verification of the reproducibility of such phenomena is generally lacking One problem is that in the absence of an identifiable and measurable specific mechanism of action the production of microbial interference is an empiric phenomenon without a clear basis for either prediction measurement or precise experimental verification Furthermore ecosystems tend to revert to their normal pattern when outside influences are removed so that the effects produced by microbial interference are generally transient and difficult to sustain

Nevertheless a few striking instances of the application of this principle exist The most recently documented example is the implantation in newborn infants in hospital nurseries of a strain of *Staphylococcus aureus* selected for its high susceptibility to penicillin and apparent lack of invasiveness in an attempt to prevent colonization with more pathogenic staphylococci (Shinefield *et al* 1963 Boris *et al* 1963 Cohen *et al* 1963)

Interferon a host tissue substance which has received wide attention in recent years represents a wholly different phenomenon wherein virus infected host cells produce a substance that conveys resistance to viral infections in other cells This effect which has not been recognized in bacterial infections has been well summarized by Grossberg (1963)

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40

Treatment of Infectious Diseases

INTRODUCTION

Spectacular discoveries and developments of the past three decades have provided clinicians with several effective forms of treatment for many common and important kinds of infectious disease The present chapter offers a general survey of these different methods of therapy Antimicrobial drugs receive the major emphasis because of their vast relative importance in clinical medicine today nevertheless it is emphasized that other means of therapy especially surgical procedures can also be highly effective Little attention has been given here to diseases caused by viruses protozoa or helminths because they are not within the scope of the volume Also the subject of *prophylaxis* of infectious disease in man is covered in another chapter

ANTIMICROBIAL DRUGS

Terms Employed in Connection With the Use of Antimicrobial Drugs

Antimicrobial literally against microbes is an excellent word and is usually to be preferred to other synonyms The word *antibiotic* literally against life was originally coined to indicate a product of a living cell antagonistic to other living cells Many of these substances have since been chemically defined and some are now prepared partially or wholly by chemical means The word *chemotherapy* is often used as synonymous with antimicrobial therapy and has an advantage over the expression antibiotic therapy because it also encompasses use of such non microbially derived substances as the sulfonamides and isoniazid Ordinary usage avoids the expression chemotherapy in connection with most uses of chemical agents with one exception cancer chemotherapy *Antiseptic* means against infection or against putrefaction This term is usually employed in connection with compounds too poisonous to be given systemically but capable of destroying germs when applied to the surface of the human body

Historical Review

The modern era of antimicrobial therapy began about 1935 with the finding that sulfonamide compounds were capable of suppressing infections caused by beta hemolytic streptococcus meningococcus gonococcus and some gram negative bacilli During the next few years modifications of the sulfonamide structure provided additional agents which were effective against the pneumococcus and the staphylococcus Penicillin was discovered by Fleming in 1928 However it was not until the 1940 s that a major effort was made to prepare it on a large scale for clinical use for the treatment of wound infections incurred in the course of war By 1943 its clinical effectiveness particularly in human staphylococcal infections had been established The problems of mass production were solved rapidly so that by 1946

supplies were abundant and the cost quite acceptable Penicillin proved to be generally superior to sulfonamides in the treatment of meningococcal gonococcal pneumococcal staphylococcal and streptococcal infections (including bacterial endocarditis) In addition it was found to be far superior to arsenicals in the treatment of syphilis Success with penicillin prompted a world wide search for other antimicrobials The first major find was streptomycin antagonistic to many gram negative bacteria and also—of particular importance—to the tubercle bacillus it was followed shortly after by the broad spectrum antibiotics the tetracyclines and chloramphenicol In the late 1940 s para amino salicylic acid was found to be useful in the treatment of tuberculosis About the same time another chemical agent isoniazid was introduced this drug has proved to be most effective and useful in the treatment of tuberculosis During the 1950 s many other new antimicrobial agents were given clinical trial and several proved to have definite value erythromycin bacitracin polymyxin vancomycin and neomycin Late in that decade came the first really effective agent for the treatment of systemic fungal infections in man amphotericin B In the first years of the 1960 s the major advance has been production of semisynthetic penicillins some of which have been of great value in treating infections caused by penicillinase producing staphylococci some also have limited value against gram negative bacillary infections Lastly in the present decade there has been introduced the first effective agent for human disease caused by a small virus i e iododeoxyuridine for keratitis due to herpes simplex virus

The Impact of Antimicrobial Drugs on Clinical Medicine

The widespread use of the various compounds mentioned in the foregoing paragraph has altered the importance of many human diseases and changed patterns of medical care Bacterial endocarditis miliary tuberculosis and cryptococcal meningitis formerly regarded as practically always fatal are now curable Many other diseases which at one time were associated with substantial fatality rates such as lobar pneumonia acute osteomyelitis tularemia typhus fever meningococcal meningitis puerperal sepsis and streptococcal septicemia now usually respond promptly to treatment Some formerly commonplace surgical operations are now rarely necessary examples are mastoidectomy or drainage of pleural empyema The therapy of syphilis has been so simplified and improved that the specialty of syphilology has disappeared and late manifestations of that disease such as gumma aneurysm and tabes dorsalis are rarely encountered Sanatoria for the care of tuberculosis have diminished in number and few physicians now devote themselves exclusively to the treatment of tuberculosis

It would be improper to conclude this brief summary of triumphs without acknowledging that there has been some cost Serious untoward effects of antimicrobial treatment have occurred and will continue Some of these will be indicated later in this chapter

The Effect of Antimicrobial Drugs on the Course of an Established Infection

How do we envisage the course of events as we watch fever and the local manifestations of an acute infection subside under the influence of an appropriate chemotherapeutic agent? There is no reason to believe that antimicrobial drugs act by enhancing host defense mechanisms they certainly exert their effects on the parasite Our knowledge of the specific biochemical events involved in the effect of the drug on the parasite is discussed in another chapter in this book For our purposes it suffices to say that the usual effect of the antimicrobial drugs is to *retard multiplication of the parasites* This usually serves to tip the balance in the host vs parasite engagement in favor of the host Final destruction and disposal of the parasites is accomplished by means of phagocytosis assisted sometimes by humoral antimicrobial substances Under some circumstances an antimicrobial drug may exert a bactericidal effect just as it can in vitro but this is probably much less common than bacteriostasis

Effectiveness of Drugs in Relation to the Character of the Infectious Lesion

Extensive clinical experience supported by experiments in animals has taught us

that antimicrobial therapy is more effective in certain kinds of lesions than in others A good example may be seen in pneumococcal infections of various parts of the body Pneumococcal pneumonia is easy to control with comparatively small doses of penicillin as little as 50 000 units of penicillin per day for 4 to 5 days has been shown to be sufficient for arrest of this process in most patients (Such small doses however are not employed in present-day clinical practice) Higher daily dosage and considerably longer treatment are needed to eradicate pneumococcal empyema or pericarditis Pneumococcal endocarditis or meningitis is now usually treated with doses in the range of 12 to 30 million units per day for 3 to 6 weeks Thus the same organism with exactly the same in vitro sensitivity to the drug may require far higher daily doses and for longer periods depending on the character of the pathologic lesion As a general rule we may say that abscesses containing sizeable quantities of purulent material are difficult to sterilize with drugs alone Experimental investigation indicates that the ineffectiveness of drug therapy directed against abscesses is related to inadequate supply of fresh viable leukocytes and to the physiologic state of organisms as they exist in the exudate An additional factor which seems to be of secondary importance may be delayed diffusion of the drug through the wall of the cavity and into the exudate The basis for difficulty in treating meningitis stems partly from impaired diffusion of drugs across the blood brain and the blood spinal fluid barriers but in addition conditions for effective phagocytosis are probably less favorable in spinal fluid than in most tissues The problem in the treatment of bacterial endocarditis appears to be the protected location of bacteria inside a fine meshwork of fibrin and platelets where they are almost inaccessible to leukocytes

General Host Factors and Effectiveness of Antimicrobial Drug Therapy

It is well known that some disease states are associated with increased susceptibility to certain kinds of infection and that antimicrobial therapy may be less effective in eradicating infections in these patients Severe granulocytopenia may be cited as an example Here there can be little doubt that the reduced number of effective phagocytes available for delivery at the site of an infectious process makes drug therapy less effective States in which there is defective antibody production such as agammaglobulinemia multiple myeloma and chronic lymphocytic leukemia are associated with increased incidence of pyogenic infection and sometimes with poor response to treatment Various systemic diseases such as uremia diabetes mellitus and acute leukemia are notoriously liable to infection with impaired ability to respond to therapy Localized increases in tissue pressure may favor the development and the persistence of infection This is a common consequence of obstruction of excretory ducts as in the urinary or the biliary tracts Other treatments as with steroid hormones antimetabolites or irradiation render patients less able to benefit from antimicrobial drug therapy than persons otherwise in good health

The Phenomena of Relapse and Microbial Persistence

Relapse may occur after apparent clinical improvement with all kinds of antimicrobial drug treatment The assumption is that clinical relapses are due to persistence of some infecting microbes in a state insusceptible to drug action or to their residence in a protected location for example in an intracellular site When therapy ceases the surviving microbes then begin to multiply again and to bring about manifestations of infection As a result of much clinical experience certain rules of practice have been established more or less empirically to govern the length of time therapy should be continued with a view to reduction of the relapse rate to a tolerable figure In some infections such as pneumococcal pneumonia or gonococcal urethritis relapse after clinical improvement is comparatively uncommon whereas in typhoid fever relapse is very likely unless treatment is continued for at least 2 weeks

Microbial persistence has been defined by McDermott as the capacity of microbes to survive drug exposure in the tissues despite susceptibility to the drug in vitro It constitutes a serious clinical problem in at least 3 infections syphilis tuberculosis and staphylococcosis Here microbial persistence may

express itself by an exacerbation of infection months or even years after apparent eradication The same organism apparently still susceptible to the drug which was used in therapy is again demonstrable in the patient s tissues Study of this phenomenon seems to yield evidence that the organisms are capable of assuming different forms perhaps some which we cannot even recognize during therapy and for some weeks thereafter but later have the capacity to revert to their original morphologic and physiologic states setting up the infectious process anew

Various practical maneuvers have been designed to cope with the problems of relapse and microbial persistence With the exception of gonococcal infections it is standard practice to continue treatment of almost all infections for 3 to 6 days after clinical improvement begins Typhoid fever staphylococcal infections and bacterial endocarditis are nearly always treated for 2 to 8 weeks regardless of how quickly the clinical signs may disappear after the beginning of treatment The longest courses of therapy are employed in tuberculosis and fungal diseases in which treatment is usually maintained for months after clinical remission It is the usual practice in such cases to arrange for follow up observations long after cessation of treatment

Acquisition of Drug Resistance During Therapy

Some pathogenic microorganisms have the capacity to develop resistance to an antimicrobial agent during the course of therapy Present evidence supports the concept that this is due to spontaneous occurrence of drug resistant mutant strains which are able to reproduce even in the presence of the drug whereas drug susceptible strains are suppressed and destroyed The phenomenon of emergence of drug resistant forms is observed relatively commonly with all antimicrobial drugs except penicillin and chloramphenicol Combined drug therapy i e the administration of two or more antimicrobial agents is employed widely to prevent or retard the emergence of resistant variants

Variable Prevalence of Drug resistant Strains

Throughout the world it has been observed repeatedly that therapy with certain antimicrobial drugs leads to an increased incidence of infections by pathogenic organisms resistant to those drugs For example when penicillin was first introduced in the mid 1940 s only about 10 per cent of staphylococcal infections were caused by penicillinase producing i e resistant strains With wide availability and use of the drug the proportion of penicillinase producers has increased so that in the general population of the United States the incidence is now something like 30 to 40 per cent and in hospital acquired infections the incidence of resistant strains may be as high as 70 or 80 per cent Similar trends have been observed frequently in connection with gram negative bacillary infections of the urinary tract These are frequently hospital acquired and a correlation often exists between the incidence of drug resistant coliform organisms and most popular antimicrobial agents With curtailment of the use of an antibiotic in an institution or a community a progressive return of drug sensitive strains can be observed during succeeding months

Superinfection

The term superinfection is employed to designate a well known and not uncommon complication of antimicrobial therapy the development of a secondary infection due to another organism which is insusceptible to the agent being administered An example would be the occurrence of klebsiella pneumonia while penicillin is being administered to a patient with a pneumococcal infection The phenomenon of superinfection is important in clinical practice the physician must be alert to recognize the development of new infectious processes during therapy There can be little question that the existence of the normal flora in the mouth the upper respiratory tract and the intestinal tract of man tends to prevent or retard the growth of various other organisms which may be introduced into these areas However when an antimicrobial drug is given and the growth of normal flora is interfered with some drug resistant organism which previously had been held in check by the equilibrium of normal coexistence now has an opportunity for unrestricted growth Then the newly dominant strain may be capable

of invading tissues and causing clinical dis ease Such infections are called endoge nous They may be extremely serious as for example the gram negative sepsis likely to occur after 2 or 3 weeks in severely burned patients receiving penicillin or the deep in fections of the mouth or the peri anal area in patients with leukemia who are under treatment with broad spectrum antibiotics This complication caused by upsetting the normal equilibrium of the patient s flora is one of the strong arguments against the in discriminate use of prophylac ic chemother apy The patient who has suffered a cerebro vascular accident may be better off with the risk of pneumonia caused by a pneumococ cus normally present in his nasopharynx than with the risk posed by prophylactic chemotherapy which renders him susceptible to pneumonia caused by for example a resistant strain of staphylococcus The fungal infections such as moniliasis following pro longed courses of antimicrobial therapy seem to be additional instances of superin fection whereby fungi of wide prevalence but generally low virulence are able to invade and cause disease when normal microbial inter relationships are disturbed by chemo therapy

Bactericidal vs Bacteriostatic Drugs

As judged by in vitro experiments some antimicrobial drugs in concentrations which can be achieved with safety in clinical prac tice are capable of outright killing of sus ceptible bacteria whereas others only retard the rate of growth but do not kill The first class i e bactericidal includes penicillin streptomycin vancomycin and bacitracin whereas the so-called broad-spectrum drugs such as sulfonamides tetracyclines chloram phenicol and erythromycin generally appear to be only bacteriostatic in therapeutic levels However drug action in vivo may differ from that observed in the test tube For example meningococcus or shigella organ isms are rapidly destroyed in the body during sulfonamide therapy whereas this drug in vitro usually exerts only a bacteriostatic action

In clinical usage the tendency is to em ploy bactericidal agents preferentially in in fections where host defense mechanisms do not function well Thus in bacterial endo carditis clinical experience has shown that bacteriostatic agents such as the tetracyclines and chloramphenicol although apparently effective as judged by in vitro testing are almost never curative Penicillin on the other hand seems to be capable of bringing about the destruction of all bacteria in the vegetation over a period of time To some extent this principle also guides the choice of drugs in the treatment of meningitis or urinary tract infection

Factors Affecting Dosage and Frequency of Drug Administration

The pharmacologic properties of various antimicrobial agents are easily studied in animals and man and the peculiarities of the various agents are well known Absorp tion from the gastrointestinal tract inactiva tion by stomach acid local irritating effect in muscle or subcutaneous tissue binding to tissue proteins conjugation or degradation rate of excretion etc all have a bearing on practice regarding methods of administration and dosage For example penicillin is rapidly excreted by the kidney thus it must be administered in large quantities and in a manner which provides for steady entry of new supplies into the circulation In sulfona mide therapy the usual practice is to give an initial loading dose in order to achieve a desired tissue level then a smaller quantity every 4 to 8 hours to maintain that level Since most antibiotics are eliminated from the circulation fairly rapidly loading doses are not given In therapy of infections caused by microorganisms that grow comparatively slowly such as the tubercle bacillus or some fungi administration of an antimicrobial agent can be spaced at wide intervals that is once a day or even once in 2 or 3 days whereas it is generally felt that for organisms which multiply rapidly such as meningococ cus or pneumococcus frequent drug ad ministration and continuous suppressive levels may be needed A matter of current debate is whether one should aim for the maintenance of high continuous levels of the drug in the management of some acute infec tions or whether it is preferable to create fluctuating drug levels in order to permit organisms to enter from time to time growth phases in which they are more drug sus ceptible Clinical evidence does not prove

express itself by an exacerbation of infection months or even years after apparent eradication The same organism apparently still susceptible to the drug which was used in therapy is again demonstrable in the patient's tissues Study of this phenomenon seems to yield evidence that the organisms are capable of assuming different forms perhaps some which we cannot even recognize during therapy and for some weeks thereafter but later have the capacity to revert to their original morphologic and physiologic states setting up the infectious process anew

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recurrent streptococcal infection in rheumatic fever subjects prevention of venereal disease in individuals about to be or very recently exposed and termination of epidemics of meningococcal meningitis or shigella dysentery in closed populations such as military groups or school children

Chemoprophylaxis is also often practiced in dealing with patients who have chronic bronchitis or fibrocystic disease in the hope of minimizing flare ups of respiratory infections In such patients recurrences of infections due to pneumococcus or *Hemophilus influenzae* may be diminished but there also is some danger of inducing a superinfection caused by a resistant microorganism

Chemoprophylaxis has been employed extensively in many kinds of surgery Unquestionably surgery of the lung has been made substantially safer because development of mediastinitis or empyema can be largely prevented by chemotherapy at the time of operation Some surgeons believe that the same holds true of intestinal surgery However there is room for question about the advisability of prophylactic antimicrobial drug therapy in clean surgery such as hernia repair or an orthopedic procedure In these situations where the likelihood of postoperative infection is slight the risk of superinfection and all the other hazards of chemotherapy may be greater than the potential benefit

The main abuse of prophylactic chemotherapy—and it is a massive one—is in giving antimicrobial agents to persons with common respiratory disease or other viral infections such as measles or poliomyelitis The explanation offered to justify such therapy is that secondary bacterial infection can be prevented by the use of antimicrobial agents The fact is that in every carefully controlled test the anticipated benefit from antimicrobial therapy has not been achieved Patients who receive prophylactic chemotherapy develop bacterial infections with the same or greater frequency and in addition the infecting organisms tend to be species insusceptible to the drug being used A similar situation exists in connection with prophylactic chemotherapy for patients who must have an indwelling catheter in the urinary bladder Because of the open communication provided by the sheath of exudate which surrounds an indwelling catheter these patients are almost certain to develop some kind of bacterial invasion of the bladder urine Prophylactic chemotherapy in this setting serves only to encourage the development of infection by resistant organisms

The following opinions regarding prophylactic chemotherapy are offered This form of treatment is often successful in preventing invasion by specific microorganisms such as the gonococcus or the hemolytic streptococcus It may also prevent the development of infection in areas which have been contaminated on a single occasion as in the course of a surgical procedure Prophylactic chemotherapy cannot be expected to maintain a microbial vacuum in areas such as the respiratory tract or the urinary bladder containing an indwelling catheter In these latter examples the patient s best interest is served by permitting bacteria normally dwelling on his body surfaces to maintain their dominance in preference to fostering the emergence of microbial species against which the host may have less effective defences and for which antimicrobial chemotherapy may be less helpful

Untoward Effects of Antimicrobial Agents

The induction of superinfections and endogenous infections during drug therapy has already been described as a serious consequence of antimicrobial therapy In addition the various drugs cause a wide variety of untoward effects involving literally every organ system in the body These vary in gravity from merely troublesome to life threatening Some appear to be direct toxic effects while the majority appear to be various kinds of allergic reactions Only a few examples will be listed here but it must be emphasized that the sum of all the complications of antibiotic therapy constitutes a substantial medical problem in the United States today Among the toxic effects may be mentioned 8th nerve damage by streptomycin rare but potentially serious bone marrow suppression by chloramphenicol injury of the liver by the tetracyclines and chloramphenicol (in newborn infants) and injury of the kidney by neomycin, bacitracin or am

one method to be clearly preferable to the other and animal experiments have yielded differing results The trend in clinical practice seems to be in the direction of maintaining high constant levels in preference to widely fluctuating drug concentrations

Use of Drug Combinations

In spite of the attractiveness of treating each infection with one magic bullet there is a tendency in clinical practice to use 2 or more antimicrobial agents in the management of many infectious diseases Sometimes this is necessitated by uncertainty regarding the microbial etiology of the given infection as is likely in the case of infections which result from perforation of the intestine, where the etiology is almost certainly complex Another basis for administering more than one agent is the possibility of achieving an additive or synergistic action against an organism Such effects have been demonstrated repeatedly in the laboratory and at times also in clinical practice The best example is the use of a combination of penicillin and streptomycin in enterococcal bacterial endocarditis The enterococcus in vitro is almost totally resistant to streptomycin and is comparatively resistant to penicillin nevertheless by administering both drugs in enterococcal endocarditis eradication of the bacterial population may be achieved Another indication for the administration of a second antibiotic has been mentioned previously namely to delay the emergence of resistant mutants

In many clinical situations however treatment with a single antimicrobial drug is entirely satisfactory e g penicillin for pneumococcal pneumonia In these instances combinations of drugs should be avoided so as to minimize the risk of direct toxicity or indirect complications caused by the therapeutic agents

The Use of In Vitro Sensitivity Tests

The physician must often depend on laboratory tests of drug sensitivity in selecting the best agent for a particular problem In some situations laboratory assistance is essential to proper choice of therapy, e g in infections of the urinary tract, staphylococcal infections and bacterial endocarditis Clinical use of sulfonamides is handicapped because of technical difficulty in carrying out such tests Pneumococci and group A beta hemolytic streptococci are so uniform in sensitivity to penicillin that laboratory confirmation is not required Sensitivity testing is not practical or necessary in the treatment of syphilis and is too slow to be of assistance in beginning treatment for tuberculosis

Serious limitations of sensitivity tests in addition to those already indicated should be noted The method most commonly employed in clinical laboratories is to observe the zone of growth inhibition on an agar medium around a disk impregnated with the drug in question The width of this zone varies not only according to the drug s activity but also in relation to its diffusibility in agar Such a test fails to provide information regarding bactericidal action Furthermore it must be interpreted in relation to the permissible dose range a factor of considerable importance when we take note of the strict dosage limitations possible with drugs such as the tetracyclines in contrast with the almost unlimited quantity of penicillin a patient can tolerate Determination of the actual drug concentration needed for bacteriostatic or bactericidal action can be carried out by incubation of the organism in a series of test tubes containing different quantities of drug This method is tedious, but it has some clinical usefulness as a guide to necessary dosage It can also be helpful in indicating possible synergism or antagonism of 2 or more agents though there are practical limitations to the number of combinations in varying concentrations that can be tested this way It should also be kept in mind that results of drug sensitivity tests done in vitro may not reflect quantitatively or qualitatively the interaction between the antimicrobial agent and the parasite in the host tissues

Prophylactic Use of Antimicrobial Drugs

Prevention of infection by use of antimicrobial drugs is discussed in another chapter nevertheless it is a subject deserving at least brief consideration here Chemoprophylaxis has been remarkably successful in situations in which infection by a specific microorganism is feared Examples are prevention of

Immobilization

The dispersion of pathogenic microorganisms in tissue can be greatly retarded if the infected area is put at complete rest Sometimes this can be achieved when the contaminated area can be immobilized by application of a plaster cast Under such circumstances in spite of the presence of pathogenic microorganisms in the area of injury as usually accompanies bullet wounds there may be little or no spread of bacteria into adjacent tissues This treatment is often of considerable value in the management of localized infections of the extremities

Relief of Obstruction

There is abundant evidence that pyogenic infections tend to develop whenever the excretory duct of an organ is occluded Such lesions may respond little or not at all to antimicrobial drug therapy This difficulty is encountered in many situations urinary tract obstruction biliary tract obstruction bronchial obstruction or paranasal sinus obstruction In most instances all that is needed in these patients is to relieve the obstruction natural defense mechanisms then seem to be capable of dealing with the infectious agent The mechanism by which obstruction enhances the progress of an infection seems to be not merely by causing a stagnant collection of fluid in which organisms can proliferate luxuriantly There is much basis for the belief that increased pressure resulting from the obstruction interferes with host defense mechanisms Experiments in animals with ureteral or biliary tract obstructions appear to show that the initial bacterial growth takes place in the compressed tissues rather than in the stagnant fluid

Of course relief of increased tissue pressure may be at least partly responsible for the beneficial effect of incision and drainage on localized collections of pus Indubitably remarkable increases in tissue pressure may accompany acute inflammation Clinical evidences of this are seen in the spurting of pus following incision the bulging of an inflamed joint the stripping of periosteum off the surface of a bone etc These local increases in pressure which accompany acute inflammation may serve to impede local defense mechanisms the precise nature of the alterations in local defense agencies is not known

SERUM THERAPY

At present serum treatment of infectious disease is restricted largely to infections in which serious or fatal injury is due to exotoxins produced by the bacteria responsible for diphtheria tetanus and botulism The capacity of specific antibody to neutralize these toxins is thoroughly demonstrated and the clinical efficacy of antiserum in these situations seems to be beyond question

Serum therapy has also been shown to be highly effective in certain acute infectious processes notably pneumococcal pneumonia and *H influenzae* meningitis It is probably significant that these infections are caused by bacteria possessing a polysaccharide capsular substance Very probably the mechanism of beneficial effect is coating of capsular material by antibody facilitating phagocytosis of the bacteria These forms of serum therapy have largely gone out of style because drugs are as effective and far simpler to use

Although antibodies are of great practical importance in the prevention of viral disease they have been notably unsuccessful in the treatment of most viral infections Certain qualifications of the foregoing statement will be found in the succeeding section on the use of gamma globulin

HUMAN GAMMA GLOBULIN

The main use of human gamma globulin at present is in prophylaxis against certain infections caused or presumed to be caused by viruses especially infectious hepatitis or serum hepatitis There is evidence that it may be of some value in the treatment of certain serious forms of vaccinia and that it may reduce the incidence of orchitis in adult males who have mumps parotitis Gamma globulin has also been used widely to produce an attenuated disease in susceptible children exposed to measles Finally to be mentioned is the value of gamma globulin administration in alleviating at least temporarily the susceptibility to certain infectious diseases of persons afflicted with agammaglobulinemia

photericin B Examples of drug allergy are fever skin eruptions and serum type sickness Various kinds of hematopoietic disorders associated with antimicrobial drug therapy such as hemolytic anemia thrombocytopenia and granulopenia may be other forms of hypersensitivity reaction or they may be expressions of a genetic abnormality analogous to the hemolytic anemia which appears during antimalarial therapy in persons with glucose 6 phosphate-dehydrogenase deficiency Penicillin is unique among pharmacologic agents in that it can be tolerated in quantities exceeding those of almost any other known chemical without evidence of direct injurious effect nevertheless penicillin can induce exceedingly serious allergic reactions anaphylaxis bullous or exfoliative skin eruptions and generalized vasculitis Such reactions have been the cause of hundreds of deaths

Sometimes new expressions of drug toxicity are recognized only after prolonged experience for example the finding after more than a decade of use that the tetracyclines seem to produce a general catabolic effect with an increase in the level of blood urea nitrogen in patients with diminished renal reserve also the tendency of the tetracyclines to produce discoloration of the teeth in children

Some Misuses of Antimicrobial Agents

In view of the dangers as well as the cost of antimicrobial drugs it is important to emphasize here the improper uses made of them In the United States antimicrobials constitute a substantial proportion of all drugs prescribed and of the nation s total expenditure for therapy Unfortunately a sizeable proportion of this is for insufficient reason or is wrong! At the top of the list is the widespread practice of employing antimicrobials in the treatment of common respiratory disease which has already been condemned Second is the tendency to prescribe an antimicrobial drug for any patient with a fever Too often the symptom is caused by some process not susceptible to this therapy e g a viral disease or a non infectious febrile disorder In some instances the patient may be suffering from an infection for which appropriate therapy is available but the wrong drug may be chosen Certain faults may be mentioned in the manner of conducting therapeutic trials The practice itself i e the giving of a drug for a diagnosis suspected but not proved is defensible The faults lie either in undue prolongation of the trial or in changing from one drug to another too quickly Another error in chemotherapy less frequent but nevertheless serious is failure to recognize the development of a drug fever this may lead to the erroneous conclusion that the original infection persists A final point to be mentioned here is failure to employ other measures to treat infection In particular this applies to surgical measures which may be essential

SURGICAL MEASURES

Evacuation of Pus

A collection of pus which can be approached surgically with reasonable safety should always be drained In view of the relative ineffectiveness of antimicrobial drugs in combatting germs contained in a large collection of pus the mechanical removal of exudate often determines success or failure It is desirable that the drainage be done while the patient is receiving appropriate antimicrobial therapy indeed it may be advisable to wait until the drug has had an opportunity to keep the infectious process contained for a few days before the surgical procedure is carried out

Removal of Dead Tissue or Foreign Body

In certain infections particularly staphylococcal osteomyelitis large chunks of dead bone may lie within the area of infection These provide excellent harbors for bacterial persistence in areas to which leukocytes can not gain access The complete cure of chronic osteomyelitis can seldom be accomplished with drugs alone but is frequently possible after the dead infected bone has been removed Other foreign bodies such as metal plates and screws silk sutures and wood splinters may have to be eliminated surgically in order to bring about eradication of an infection

Immobilization

The dispersion of pathogenic microorganisms in tissue can be greatly retarded if the infected area is put at complete rest Sometimes this can be achieved when the contaminated area can be immobilized by application of a plaster cast Under such circumstances in spite of the presence of pathogenic microorganisms in the area of injury as usually accompanies bullet wounds there may be little or no spread of bacteria into adjacent tissues This treatment is often of considerable value in the management of localized infections of the extremities

Relief of Obstruction

There is abundant evidence that pyogenic infections tend to develop whenever the excretory duct of an organ is occluded Such lesions may respond little or not at all to antimicrobial drug therapy This difficulty is encountered in many situations urinary tract obstruction biliary tract obstruction bronchial obstruction or paranasal sinus obstruction In most instances all that is needed in these patients is to relieve the obstruction natural defense mechanisms then seem to be capable of dealing with the infectious agent The mechanism by which obstruction enhances the progress of an infection seems to be not merely by causing a stagnant collection of fluid in which organisms can proliferate luxuriantly There is much basis for the belief that increased pressure resulting from the obstruction interferes with host defense mechanisms Experiments in animals with ureteral or biliary tract obstructions appear to show that the initial bacterial growth takes place in the compressed tissues rather than in the stagnant fluid

Of course relief of increased tissue pressure may be at least partly responsible for the beneficial effect of incision and drainage on localized collections of pus Indubitably remarkable increases in tissue pressure may accompany acute inflammation Clinical evidences of this are seen in the spurting of pus following incision the bulging of an inflamed joint the stripping of periosteum off the surface of a bone etc These local increases in pressure which accompany acute inflammation may serve to impede local defense mechanisms the precise nature of the alterations in local defense agencies is not known

SERUM THERAPY

At present serum treatment of infectious disease is restricted largely to infections in which serious or fatal injury is due to exotoxins produced by the bacteria responsible for diphtheria tetanus and botulism The capacity of specific antibody to neutralize these toxins is thoroughly demonstrated and the clinical efficacy of antiserum in these situations seems to be beyond question

Serum therapy has also been shown to be highly effective in certain acute infectious processes notably pneumococcal pneumonia and *H influenzae* meningitis It is probably significant that these infections are caused by bacteria possessing a polysaccharide capsular substance Very probably the mechanism of beneficial effect is coating of capsular material by antibody facilitating phagocytosis of the bacteria These forms of serum therapy have largely gone out of style because drugs are as effective and far simpler to use

Although antibodies are of great practical importance in the prevention of viral disease they have been notably unsuccessful in the treatment of most viral infections Certain qualifications of the foregoing statement will be found in the succeeding section on the use of gamma globulin

HUMAN GAMMA GLOBULIN

The main use of human gamma globulin at present is in prophylaxis against certain infections caused or presumed to be caused by viruses especially infectious hepatitis or serum hepatitis There is evidence that it may be of some value in the treatment of certain serious forms of vaccinia and that it may reduce the incidence of orchitis in adult males who have mumps parotitis Gamma globulin has also been used widely to produce an attenuated disease in susceptible children exposed to measles Finally to be mentioned is the value of gamma globulin administration in alleviating at least temporarily the susceptibility to certain infectious diseases of persons afflicted with agammaglobulinemia

FEVER THERAPY

A question frequently asked is whether fever constitutes an important defense mechanism against infectious disease As a general rule elevation of body temperature does not seem to play a significant role in the control of microbial infections Nevertheless it is worth noting that some strains of type III pneumococcus and of cryptococcus are unable to cause severe infections in animals capable of a normal febrile response whereas they are able to proliferate rapidly in animals whose temperatures are held down by antipyretic drugs

In clinical experience one infectious disease is known to respond favorably to fever therapy namely the form of neurosyphilis known as general paresis Fever treatment is resorted to only occasionally now because good results are obtained from the simpler and safer treatment by penicillin Nevertheless it is important to point out that in the years before penicillin was available fever therapy was highly successful in treating an otherwise progressive and often fatal form of syphilitic infection Methods of fever therapy which were employed in neurosyphilis included induction of malaria intravenous injection of bacterial endotoxin and elevation of the body temperature by treatment in a radiant heat cabinet The mechanism by which fever therapy was so effective in this disease has never been established with certainty Possibilities include deleterious effect on the parasite due to the higher temperature or enhancement of host defense mechanisms through stimulation of the reticuloendothelial system

STEROIDS AND ACTH

Controversy exists regarding the advisability of using adrenocorticotropic hormone (ACTH) or adrenal steroid compounds in the treatment of certain kinds of infection The unquestionable anti inflammatory action of many of these may appear to reduce the manifestations of inflammation lowering the body temperature diminishing local pain and lessening malaise On the other side of the debate must be considered the possible danger of ACTH and steroid therapy Ample experimental and clinical evidence shows that treatment with these compounds may actually accelerate the progress of infection leading to widespread dissemination of the infectious agent and a higher death rate

At times the improvement in symptoms and signs due to diminution of the acute inflammation is indeed clinically impressive Some experienced clinicians believe that this form of therapy may be life saving when used in conjunction with appropriate antimicrobial drugs particularly in the management of overwhelming infections The reasoning is that by diminishing the toxic manifestations of the rampant infection survival may be prolonged sufficiently to permit an antimicrobial drug to gain control of the infectious process The use of steroids in conjunction with chloramphenicol in the treatment of typhoid fever or severe brucellosis is advocated for the purpose of hastening defervescence of fever and preventing a Herxheimerlike effect resulting from too rapid destruction of the parasite by the antimicrobial drug Steroids are sometimes employed in the management of severe inflammatory processes due to viruses or presumed viruses as for example in the management of overwhelming varicella pneumonia or severe infectious mononucleosis

Those who oppose the use of ACTH and steroids cite the extensive experimental evidence showing enhanced spread of infection in animals receiving these hormones as well as the demonstrated diminution of immune responses that they produce They cite too the experimental evidence that steroids may enhance the injurious effects of bacterial endotoxin such as renal cortical necrosis

The truth is difficult to discern in the controversy regarding clinical evaluation of ACTH or steroid therapy In one cooperative clinical study involving a double blind test of cortisone therapy in patients with severe bacterial infections the results showed little difference either of benefit or harm from the hormone Despite frequent temporary improvement in clinical appearance following ACTH or steroid therapy there is no convincing proof that it is ever life saving and there is a lessening tendency among clinicians to employ this kind of treatment

GENERAL SUPPORTIVE CARE

Unquestionably one of the oldest methods of treating infectious disease—bed rest—is generally beneficial Why this should be can not be stated very accurately but it is usually assumed that lessening the work of the heart the lungs and the kidneys augmenting blood flow to the liver and reducing caloric re quirements are all in the best interest of the acutely ill patient Good supportive care in addition to bed rest i e administration of adequate fluids salt and vitamins by paren teral routes if need be has certainly been of value in the management of prolonged febrile illness

Although again the mechanism is not clear clinical experience seems to provide undeniable evidence that measures to correct acidosis and azotemia are helpful in assisting patients to control infectious processes

The use of antipyretic agents to lower the temperature level is much debated clinically Probably this is not of vital importance in most instances Some patients experience great malaise in the presence of fever others are scarcely aware of temperature elevation In the present era of antimicrobial drug therapy the response of the body tempera ture is one of the most useful guides in judg ing success or failure of therapy and because of that the use of antipyretics may be unde sirable Often malaise can be controlled with a drug such as codeine which does not also have an antipyretic effect

Interest is developing in the question of whether benefit may be achieved in certain cases by lowering body temperature to sub normal levels as can be accomplished by mechanical cooling devices This is being recommended especially in the treatment of bacteremia due to gram negative bacilli Cases have been reported in which the cir culatory shock of such severe infections has subsided only after the patient s body tem perature had been lowered to the range of 80 to 85 F This therapy is still in the stage of trial undoubtedly involves some danger and requires most careful evaluation by qualified observers nevertheless eventu ally it may prove to have therapeutic value in selected instances

In the foregoing survey an attempt has been made to present very generally the current situation in clinical management of infectious diseases Spectacular progress has been achieved though not without some cost e g drug toxicity superinfections We still lack a specific treatment for nearly all viral diseases But we can expect many ex citing break throughs The certain prospect for the future is progress—and new problems

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Bibliographic Index

Subject Index